The quantitative determination of phytate and available phosphorus for maize (*Zea mays* L.) breeding

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CHAPTER 1. GENERAL INTRODUCTION

The prevalence of phytate, an unavailable form of phosphorus (P), in maize grain is a source of environmental and nutritional problems (Raboy, 2002). Because many of these issues are not due to the total P concentration in grain per se and available P is an essential nutrient for livestock and humans, it would be desirable to reduce phytate and increase available P levels of maize (Raboy, 2001). Previous studies indicate that significant genetic variation with regard to P related traits exists across breeding lines and cultivars of various crop species (Maga, 1982; Raboy et al., 1984; Raboy et al., 1991; Wardyn and Russell, 2004). Thus, phytate and available P could be evaluated and bred as quantitative traits. Improvement of quantitative traits through selection is often successful, but their study requires the measurement of many individuals which cannot be achieved if the methods of evaluation are complicated and expensive (Falconer and Mackay, 1996). The primary objective of the present work was to investigate the possibility of improving phytate and available P concentrations in a maize breeding population through the use of simple and rapid protocols.

The assays used for phytate and available P evaluation in the subsequent chapters were derived from the methods of Vaintraub and Lapteva (1988) and Raboy et al. (2000), respectively. Vaintraub and Lapteva (1988) showed that the addition of a simple colorimetric reagent to unpurified grain extracts can precisely quantify relative phytate concentrations among different plant sources. Although their methods are among the most simple in the literature, their use for large-scale selection purposes in breeding populations have not been reported. Raboy et al. (2000) derived a *high inorganic P phenotype* assay from the methods of Chen et al. (1956) to qualitatively identify segregates carrying a low-

phytic acid (*lpa*) mutation, but the modification of this method for quantitative determinations of inorganic P has not been reported to the knowledge of the author. It was assumed in the present study that the inorganic P liberated upon dilute acid extraction is available P in the seed. Because selection only requires relative measurements for ranking genotypes, this assumption should be adequate for the purposes of a breeder.

Repeatability and broad-sense heritability estimates for phytate and available P were calculated and compared to those of other traits commonly measured in maize breeding programs. Phenotypic and genetic correlations among phytate, available P, and other traits important to the successful development of maize cultivars were estimated to identify any potential secondary selection responses that may require special attention. To investigate this further, several selection indices involving phytate, available P, and essential agronomic traits were derived and expected selection differentials were calculated. Finally, the proper use of experimental design and analysis can have significant impacts on the degree of genetic determination and thus the heritability of metric characters. Experimental designs and analyses commonly used by field breeders to tame heterogeneous experimental units were utilized in the laboratory to optimize the precision of these simple, but imperfect assays. The impact of these different "lab analyses" on heritability and family selections were studied for two reasons: 1) to determine appropriate resource allocation, 2) to illustrate the importance of design and analysis on selection despite the lack of obvious sources of error.

The results of the above objectives are presented among three manuscripts bound in this thesis. Each manuscript is followed by relevant tables and figures cited in the text. A literature review follows this introduction and is meant to familiarize the reader with phytate and available P in maize grain by covering some of the most relevant and popular literature.

A "general conclusions" chapter at the end was prepared to summarize the results of this work and provide recommendations to any student or investigator that continues and improves upon the methods used herein.

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CHAPTER 2. LITERATURE REVIEW

This literature review attempts to summarize the importance and possibility of developing maize populations with decreased phytic acid and increased available phosphorus concentrations. Published studies on phytic acid and total P concentration in maize grain are emphasized, but due to lack of volume in the literature, studies on other crop species are often referenced as well. A brief review of phytate and available P assays is provided at the end of this chapter to provide insight into methods used in the following chapters.

PHOSPHORUS AND PHYTIC ACID IN THE SEED

Phytic acid (*myo*-Inositol-1,2,3,4,5,6-hexakiphosphate) is ubiquitous in eukaryotic cells, abundant in pollen, roots, and tubers, and is the major storage form of phosphorus (P) in seeds (Cosgrove, 1980). Typically 65 to 80% of mature seed total P is in the form of phytic acid, which can make up one to several percent of the seed dry weight (reviewed in Raboy, 1990). Inorganic P and cellular P (a component of cellular membranes, DNA, RNA, etc.) are other forms of P found in the seed and are typically referred to as "available P" (Raboy, 2001).

During the plant life cycle, P is taken up from the soil in the form of inorganic phosphate, transported to the leaves and vegetative tissue, and remobilized to the seed during reproductive development (Liptay and Arevalo, 2000). During seed development, P is accumulated in the protein globoids where phytic acid biosynthesis takes place via a stepwise phosphorylation of *myo*-inositol, a cyclic alcohol derivative of glucose, by a series of kinases (reviewed Liptay and Arevalo, 2000; reviewed in Raboy, 2003). Evidence for the intracellular location of phytic acid intermediates and biosynthesis is limited and several

studies across a few species have reported conflicting results (Brinch-Pedersen et al., 2002). Nevertheless, the end result of the phytic acid pathway is an abundance of *myo*-inositol backbones with a phosphate group attached to each of the six carbons. In the mature seed, phytic acid is typically found bound to cations such as K^{+}, Mg^{++}, Ca^{+}, Mn^{++}, Zn^{++}, Ba^{++}, or Fe^{+++} to form a mixed cation salt known as "phytate" (reviewed in Lott et al., 2000). Cereal grains and oil seeds have particularly high concentrations of phytate (O'Dell et al., 1972) and there is no known mature seed from which it is completely absent (Lott et al., 2000).

Seed tissue-specific patterns and of phytate deposition are probably common across plant species. In corn, 88% of the phytate is found in the germ. In contrast, the aluerone layers of wheat and rice contain more than 80% of total seed phytate. Overall, due to its deposition with protein globoids, phytate is commonly associated with tissues containing high levels of protein (reviewed in Raboy et al., 1990).

Seed total P and phytic acid are highly and positively correlated so that nearly all the variation observed in total P is directly due to variation in phytic acid (Raboy, 1990). Therefore, the amount of non-phytic acid P (inorganic P and cellular P) is thought to be constant across cultivars and throughout seed development in later stages. For instance, during the first few weeks following pollination, total P begins to accumulate in the developing seed, but minor levels of phytic acid are found. Once a particular level of total P is reached later in development, all of the subsequently accumulated P in the seed is assimilated into phytic acid (Raboy, 1997).

Due to the abundance and frequency of phytic acid, and therefore phytate, in plant seeds, many hypotheses concerning its function have been proposed by researchers. Two possible major roles of seed phytate include: 1) storage of *myo*-inositol, phosphate, and

several important minerals (namely the cations mentioned above) for use by the developing seedling, and 2) the control of inorganic phosphate levels in both germinating and developing seeds (Lott et al., 2000). Both hypotheses cast phytic acid as a pool for the storage and distribution of important minerals during critical stages in the plant life cycle (reviewed by Lott et al., 2000). More specifically, one obvious role for phytic acid in seedlings is as a donor of phosphate groups for the regeneration of ATP from ADP. This mechanism was proposed during the early years of phytic acid research and was criticized subsequently, but has lately regained credibility due to further research (Raboy and Gerbasi, 1996). Accumulation of inorganic P and minerals in the seed presents challenges to the plant that are solved by sequestering these elements to phytate (Raboy, 1997). The importance of such a mechanism can be comprehended by considering a few of the many mechanisms involved in seed development and germination: 1) inorganic P regulates the enzymes important to starch and protein metabolism, 2) Ca flux is an important component to signal transduction pathways and must be maintained at specific levels against environmental concentrations, and 3) the accumulation of Fe would result in oxidative damage if there were no preventative mechanisms (reviewed in Raboy, 1997). The above summary illustrates the important role phytate plays in the development and germination of seeds. In spite of this, the discovery of low phytic acid mutants, discussed below, has suggested that an abundance of phytic acid might increase the fitness of plants in nature, but may not be critical to the normal development of cultivated crops (Raboy, 2001).

IMPACT OF PHYTIC ACID ON HUMAN AND ANIMAL NUTRITION

Phytase is a phosphatase that catalyses the hydrolysis of phytic acid to lower polyphosphates and eventually *myo*-inositol (Brinch-Pedersen et al., 2002). Phytases are widely distributed in all types of organisms including plants where they rapidly degrade phytate upon germination to release available P and other minerals (Pen et al., 1993). Phytase is also produced by yeasts and other fungi, some bird species, and ruminants (Harland and Morris, 1995). However, monogastric animals such as swine and poultry lack phytase and therefore fail to degrade phytic acid causing the abundance of P in cereal and legume based diets to be unavailable (Raboy, 2002). The addition of phytase to feed stuffs can optimize P utilization and reduce excretion into the manure (Pen et al., 1993).

Adequate P retention in monogastric animals is absolutely essential for proper growth and development. Cromwell and Coffey (1991) simply state "Phosphorus is a key element required by all living beings." As for mineral abundance in the body, P is second only to Ca and accounts for 25% of total body mineral matter (Cromwell and Coffey, 1991). Phosphorus is involved in "nearly every series of cellular biochemical reactions", is an important component of DNA, RNA, and cellular membranes, and is involved in the osmotic balance of body fluids (reviewed in Cromwell and Coffey, 1991). Spencer et al. (2000) found that dietary P level had a significant effect on daily gain, feed intake, bone ash, bone breaking load, and feed efficiency in swine. Because phytate is the main natural source of P in diets of plant origin, inorganic P must be supplemented to meet the animal's nutritional requirements (Harland and Morris, 1995). For example, the bioavailability of P in corn based diets is less than 15% (Cromwell, 1992).

The chelation properties of phytic acid are thought to have both positive and detrimental effects on human nutrition and well being. The metal binding characteristics of phytic acid may help it serve as an important antioxidant (Harland and Morris, 1995; Raboy, 2002). Whole cereals and legumes seem to provide protection against colon cancer, which is strongly influenced by dietary factors. This putative protective role offered by cereals and legumes may be due directly to their abundant phytate complexing with iron in the colon and therefore preventing the formation of hydroxyl radicals (Harland and Morris, 1995). Pretlow et al. (1992) showed that fortifying the drinking water of lab rats with phytate reduced the incidence of colon cancer from 83 to 25% when tumor cells were induced.

On the other hand, phytic acid's ability to bind with metal cations casts it as an anti-nutrient that shuttles valuable minerals out of the digestive tract. Kornegay (1996) reported that 1 mol of phytic acid can bind 3 to 6 mol of Ca to form insoluble phytate at the pH of the intestine. The availability of iron and zinc in wheat bran and whole-grain products is less than refined products (Erdman, 1981) mainly due to phytic acid's ability to irreversibly bind with these minerals in the intestinal lumen (reviewed in Mendoza et al., 1998; Raboy, 2002). Subjects fed zinc fortified rolls made from white flour (milled to remove the germ and bran that contain most of the seed phytate) retained significantly more zinc than subjects fed zinc fortified whole grain rolls (reviewed in Erdman, 1981). It has been estimated that two billion people worldwide suffer from micronutrient deficiencies (Graham and Welch, 1996) which may be partly due to an abundance of phytate in staple cereals and legumes. In general, phytic acid may have two different impacts on the lives of people in different socioeconomic classes: aging adults in developed countries may benefit from dietary phytic acid because of

its antioxidant properties, but children and pregnant women of developing nations may suffer nutrient deficiencies because of phytic acid's role as an anti-nutritional factor (Raboy, 2002).

IMPACT OF PHYTIC ACID ON THE ENVIRONMENT

Eutrophic waters are defined as being rich in organic and mineral nutrients that promote the growth of plant life, especially algae, which reduce the dissolved oxygen content and often results in the extinction of other organisms. In fresh waters, P is often the limiting nutrient to complete eutrophication (Sharpley et al., 1994). Eutrophication generally reduces the quality of surface waters for aesthetics, fisheries, recreation, industry, and drinking, which all can have a serious impact on regional economies (Sharpley et al., 1994). For example, eutrophic lakes have experienced a shift in fish populations, from white fish and pike to carp and other low-oxygen fish (Hill, 1967). Two of the Great Lakes, Lake Ontario and Lake Erie, have also shown significant decline due to eutrophication (Cromwell and Coffey, 1991). Once a body of water is eutrophic, it is difficult, time consuming, and expensive to return it to its original, clear condition (Sharpley et al., 1994). In fact, a recent study has suggested that the time required to undo the damage already done to lakes is on the order of thousands of years (Carpenter, 2005).

In addition to the problems of P nutrition associated with seed phytic acid in staple crops, pollution of surface waters (mainly eutrophication) related to P excretion into the manure of livestock is a major problem for populations in North America and Europe (Lott et al., 2000). Because the P bound in phytic acid is largely unavailable to swine and poultry, most of the P in their diets passes into the manure and onto the landscape, where it is subject to erosion and runoff (Sharpley et al., 1994). For instance, the P content in swine and poultry

manure ranges from 1.2 to 1.8%, two to three times greater than the P content of ruminant manure (Cromwell and Coffey, 1991). This contributes to the fact that swine and poultry manure account for only 18% of the total livestock manure in the USA, yet account for 33% of P excretion (Cromwell and Coffey, 1991). Historically, manure with increased levels of nutrients benefited the land through their use as a valuable fertilizer, but the intensification of single point livestock systems has resulted in manure sources containing P levels exceeding the requirements of the surrounding crops and soils (reviewed in Maguire et al., 2005). Phosphorus pollution due to this excessive generation of manure containing phytic acid is further exacerbated by the need to supplement feeds with inorganic P. Also, urban expansion has reduced the amount of land to which manure can be applied (Cromwell and Coffey, 1991). As a result, nonpoint sources (particularly agricultural runoff) of water pollution have replaced point sources as the major contributor to eutrophication (reviewed in Carpenter, 2005).

Once P is converted to its insoluble form in the soil profile, it generally immobile and does not contaminate ground water. However, because it is immobile, P builds up in the soil over time and contaminates surface water through runoff and erosion (Cromwell and Coffey, 1991). Several studies have shown that P loss in runoff is closely dependant upon surface soil P content and, for the reasons mentioned above, has increased over the years (reviewed in Sharpley et al., 1994). In 1990, an average of 48 mg kg^{-1} was present in all soils tested in Wisconsin compared to 34 mg kg^{-1} found in 1967 (Sharpley et al., 1994). After high concentrations of soil P have been reached, considerable time is required for depletion. McCollum (1991) estimated that 16 to 18 years of cropping corn and soybeans would be needed to reduce a soil P concentration of 100 mg kg^{-1} to the threshold value of 20 mg kg^{-1}.

Traditionally, manure application rates are based on nitrogen requirements with little attention paid to P concentrations in the manure or the P requirements of the crops resulting in this accumulation of soil P (Sharpley et al., 1994). Reducing the amount of unavailable P in animal diets is one option for meeting recommendations to lessen the amount of P in manures (Cromwell and Coffey, 1991).

MAIZE LOW-PHYTIC ACID MUTANT LINES

The problems associated with P in grains are not due to the concentration of total P per se, but rather to the fact the P exists in the mature seed in the form of phytic acid (Raboy, 2001). Thus, it would be desirable to increase the amount of available P and reduce the amount of phytic acid P. Low-phytic acid (*lpa*) mutant lines in maize have been developed that have normal levels of total P, but reduced levels of phytic acid and concomitant increases in either inorganic P or intermediate polyphosphates (Raboy, 2002). It is assumed that these mutations do not affect the plants ability to uptake P and distribute it to the seed, but rather interrupt the biochemical pathway responsible for the assimilation of phytic acid from phosphate (Raboy, 2002). The two lines studied most thus far are known as *lpa1-1* and *lpa2-1* and have different seed P profiles (Raboy et al., 2000). Increases in available P on the order of 5-10 fold and 3-4 fold relative to nonmutant seeds were observed for *lpa1-1* and *lpa2-1,* respectively (Raboy et al., 2000). Phytic acid in the *lpa1-1* line is reduced by 75% compared to non-mutant seeds, but in the *lpa2-1* line it is reduced by only 50% (Raboy, 2001). The reduced levels of phytic acid in the *lpa2-1* line is accompanied by both increases in inorganic P and lower level inositol phosphates, whereas the *lpa1-1* line phytic acid reduction is followed only by an increase in inorganic P (Raboy, 2001). Therefore, Raboy et

al. (2000) concluded that the lesion caused by the *lpa1-1* mutation is most likely earlier in the phytic acid pathway than the *lpa2-1* mutation. Furthermore, Raboy et al. (2001) characterized the *lpa1-1* and *lpa2-1* lines as loss-of-function mutants carrying recessive alleles at the putative *lpa* loci. In addition to *lpa1-1* and *lpa2-1*, at least 20 different mutant lines that have reductions in phytic acid ranging from 50 – 95% have been isolated and are being evaluated in breeding programs (Raboy et al., 2001).

The development of these *lpa* lines is not only a potential valuable tool for fighting the problems associated with phytic acid in maize grain, but are also valuable resources for the study of phytic acid biochemistry, physiology, and nutrition (Raboy et al., 2000; Raboy, 2001). To investigate the possibility of greater iron absorption through the consumption of maize with lowered phytic acid concentrations, Mendoza et al. (1998) measured iron absorption in human subjects that consumed two types of tortillas, one made from *lpa1-1* maize and another consisting of the wild-type counterpart. Notable conclusions include the observation that absorption of iron was increased by about 50% when the *lpa1-1* tortillas were consumed compared to the wild-type tortillas and that the authors attributed this difference to the lower phytic acid content of the *lpa1-1* tortillas (Mendoza et al., 1998). Absorption of iron from maize is reported to be lower than that of other cereals; consequently, the increased absorption from low-phytic acid tortillas implies an important contribution to populations suffering from high rates of iron deficiency (Mendoza et al., 1998).

Spencer et al. (2000) studied the effects of low-phytate corn on the digestibility and retention of P in the diets of swine. This was accomplished through comparing bone breaking load and P excretion between groups of pigs fed grain from a *lpa1-1* maize hybrid

and a wild-type, isogenic hybrid. A major conclusion by Spencer et al. (2000) was that corn with reduced phytic acid P substantially increased the P bioavailability in those diets from 0.03 to 0.17%. Additionally, pigs fed diets based on the *lpa1-1* hybrid demonstrated significantly greater P retention and reduced P excretion (Spencer et al., 2000). Thus, Spencer et al. (2000) concluded by stating that feeding low-phytate corn is a valid method for reducing the need for inorganic P supplementation and lowering the concentration of P in the feces to meet recommendations for manure nutrient levels. A similar study by Ertl et al. (1998) looked at the effect of feeding *lpa1-1* maize to broiler chicks compared to wild-type counterparts. The results were concurrent with previous reports in that the availability of P was observed to be drastically increased and the concentration of P in the feces was reduced by up to 40% (Ertl et al., 1998).

The above studies on *lpa* mutant lines poignantly illustrate the direct benefits of feeding a maize diet that has substantially reduced phytic acid P and increased available P. However, comparisons between normal and mutant lines with respect to agronomic traits important to the farmer must indicate that no difference exists for practical application of the mutant lines. Ertl et al. (1998) compared *lpa1-1* hybrids to their wild-type counterparts with respect to a series of agronomic traits. Two interesting findings by Ertl et al. (1998) include a 6% greater grain yield in the wild-type hybrids and a slightly better stand establishment among the *lpa1-1* hybrids. Because the donor parent (an *lpa1-1* line) was inferior in yield to the recurrent parent and only three backcrosses were made, the authors attribute the difference in yield to the percentage of donor parent genome still being included in the *lpa1-1* lines (theoretically 6%). However, it is possible that the yield reduction may be due to a pleiotropic effect of the *lpa1-1* mutation. One observation of Raboy et al. (2000) upon

inspection of the newly developed *lpa1-1* and *lpa2-1* lines was a reduction in seed size, which could directly affect yield potential of resulting cultivars. The greater stand establishment of *lpa1-1* hybrids reported by Ertl et al. (1998) contrasts results found in other crops in addition to speculation based upon the critical role phytic acid may play during germination. Ertl et al. (1998) did not have an explanation for this discrepancy and concluded that *lpa1-1* hybrids are not significantly different compared to their wild-type counterparts for most standard agronomic traits with yield being an exception. If yield loss is proven to be a result of pleiotropy, then other mutations or approaches to the phytic acid problems must be sought (Ertl et al., 1998).

Despite the fact that Ertl et al. (1998) reported an increase in stand establishment among the *lpa1-1* hybrids, studies on *lpa* lines in other species suggest otherwise (Hulke et al., 2004). Furthermore, the fact that an *lpa* maize hybrid has not been released by the industry alludes to the possibility that these mutations may confer some yield and germination disadvantages. An alternative approach to reducing phytic acid and increasing available P would be to initiate a selection program designed to modify these traits while applying selection pressure to other important traits to maize production in the desired directions.

GENETIC VARIANCE OF PHYTIC ACID AND PHOSPHORUS CONCENTRATION IN MAIZE GRAIN

Before the design of a selection program, it is important to survey the reported genetic variance for the traits of interest to ascertain the practicality of spending the resources required. This is realized by considering the following formulas:

$$R = h^2 S$$

$$h^2 = \frac{V_A}{V_P}$$

$$V_G = V_A + V_D + V_I$$

where R is the response to selection, h^2 is the narrow-sense heritability, S is the selection differential, V_A is the additive genetic variance, V_P is the total phenotypic variance, V_G is the total genetic variance, V_D is the dominance variance, and V_I is the epistatic variance (Bernardo, 2002). The upshot of these equations is that h^2 is needed for response to selection and adequate V_A relative to V_P is needed for h^2, which cannot exist if substantial V_G is not found. In other words, broad-sense heritability ($H^2 = V_G / V_P$) sets an upper limit to narrow-sense heritability (Falconer and Mackay, 1996).

Phytic acid and total P measurements in a variety of crops including wheat (Raboy et al., 1991), soybean (Raboy and Dickinson, 1993; Raboy et al., 1984), dry beans (Lolas and Markakis, 1975) and oat (reviewed in Maga, 1982) indicate that significant genetic variation exists for such traits. The amount of literature on the measurement of phytic acid and inorganic P in maize breeding populations to estimate potential progress from selection is scarce. Raboy et al. (1989) analyzed the 83rd generation of divergent selection in the Illinois' high and low protein lines and high and low oil lines (IHP, ILP, IHO, and ILO respectively). The whole grain phytic acid concentration in the IHP line was 18.0 mg g^{-1}, which was more than 2.5 times the concentration of the 7.0 mg g^{-1} found in the ILP line. Similarly, differences in phytic acid content between the IHO and ILO lines were significant, but less in magnitude as the IHO only had 33% higher whole grain phytic acid relative the ILO line. Although the responses of phytic acid concentration to selection observed by Raboy et al.

(1989) were secondary effects in protein and oil selection programs, their results and conclusions suggest that phytic acid can be altered through plant breeding practices. However, the differences observed in phytic acid concentration were paralleled by differences in total P, which suggests that selection was not altering the patterns and pathways responsible for synthesizing phytic acid in the seed, but rather was affecting the plants ability to uptake P in the field (Raboy et al., 1989).

Wardyn and Russell (2004a) measured total grain phosphorus concentration (P-Gr) in a set of 200 random S1 families from an Illinois Low Protein by Illinois High Protein derived population. A normal distribution of P-Gr values and a broad-sense heritability value of 0.82 was found, which indicated that substantial genetic variation exists in this population relative to environmental variation. The genotype by environmental variation was significant, but was only 18% as large as the genetic variance value. Also, Wardyn and Russell (2004) reported only a 5% loss in selection differential on average when evaluations were made over one year compared to two years. Therefore, it was concluded that single year evaluations would be adequate for selection on P-Gr and more replications per year should provide more genetic gain in time than more years and fewer replications. Although the broad-sense heritability for P-Gr calculated by Wardyn and Russell (2004) indicates that this trait should be easily modified by selection in the population they evaluated, few low P-Gr segregates indicated that many cycles of selection may be needed to improve this trait to desirable levels.

TRAIT CORRELATIONS

Identification of correlations between traits before the selection is important since the improvement of one trait may bring about simultaneous changes, desirable or undesirable, in other traits if appropriate attention is not given to the situation (Falconer and Mackay, 1996). For example, fifty generations of selection for high protein alone in Illinois resulted in lines with small ears, large cobs, and reduced yield (Woodworth et al., 1952). Phytic acid is often found to be highly correlated ($r > 0.90$) with total P (Maga, 1982; reviewed in Raboy et al., 2001). Thus, selection programs aimed at only lowering phytic acid levels are not recommended because such an approach would also likely lower total P and thus available P concentration, which is an undesirable outcome when striving for enhanced nutritional properties. Phytic acid and total P are also typically correlated with protein (Raboy et al., 1984; Raboy et al., 1991), which would be expected knowing that both compounds are most abundant in the seed embryo and aleurone layer (O'Dell, 1972). In a maize population consisting of S1 lines, a genetic correlation of 0.65 was found between grain total P and grain protein levels (Wardyn and Russell, 2004b). Raboy et al. (1989) reported that a long-term divergent selection program for protein also indirectly resulted in a 2.5 fold difference in phytic acid between the high protein and low protein lines. Due to the consistent phytic acid:protein correlations reported in the literature, the development of population or cultivar with desired levels of both of these nutritionally important traits may require evaluation of extensive amounts of breeding material and selection with a multiple trait index. Correlations between P traits and other grain quality characteristics reported in soybean include those between phytic acid and both Zn and Ca (Raboy et al., 1984). Wardyn and Russell (2004b) reported a negative association between grain yield and grain total P

suggesting that selecting for lower P levels would not adversely affect yield. No other correlations between phytic acid, inorganic P, or total P and agronomic traits are known by the author.

DETERMINATION OF PHYTIC ACID, PHYTATE, AND AVAILABLE PHOSPHORUS

The successful modification of quantitative traits via selection is contingent upon the ability to measure the traits of interest in a rapid, efficient, and reliable manner. A quote from Falconer and Mackay (1996) best describes the importance of measurement techniques in the study of quantitative characters:

> The essential condition is that they should be measurable. The technique of measurement, however, sets a practical limitation on what can be studied. Usually rather large numbers of individuals have to be measured and the study of any character whose measurement requires an elaborate technique therefore becomes impractical.

Furthermore, because the repeatability of phenotypic measurements sets an upper limit to the genetic determination and heritability of characters (Falconer and Mackay, 1996), optimizing the repeatability of the methods used is critical to the progress of breeding programs.

Selected Methods of Phytic Acid and Phytate Measurement

Many analytical techniques for the quantification of phytic acid have been based upon the observation that phytic acid forms an insoluble complex with ferric ions in dilute acid (Maga, 1982). This led to the use of the ferric precipitation method used by researchers

trying to quantify phytic acid in breeding lines or cultivars. The numerous versions of this method often include steps of extraction, purification, precipitation, centrifugation, washing of the precipitate, and wet or dry ashing of the precipitate (Harland and Oberleas, 1977; Latta and Eskin, 1980). A sample of the studies that have used a version of the ferric precipitation method for quantification of phytic acid in either wild-type or mutant breeding lines include Raboy et al. (1990), Raboy et al. (1989), Raboy et al. (2000), Raboy et al. (1991), Raboy and Dickinson (1993), Raboy et al. (1984). Although the precipitate obtained and measured using this method includes lower level polyphosphates (Maga, 1982), the measurement is valid because nearly all of the organic phosphate found in mature seeds is in the form of phytic acid. Nevertheless, assumptions of this method can lead to wide variation in results (reviewed in Maga, 1982; Talamond et al., 2000).

Haug and Lantzsch (1983) derived a similar, but indirect method of measuring phytic acid in seed extracts. The phytic acid in acid extracts was precipitated by a known quantity of acidic $Fe3^{+}$ and the decrease in $Fe3^{+}$ content was measured with a spectrophotometer upon reaction with 2,2'-bipyridine. This method avoids some steps of the ferric precipitation method, but is still laborious and requires absorbance readings to be made within a limited time frame because color changes over time may jeopardize its accuracy and precision (Vaintraub and Lapteva, 1988).

Latta and Eskin (1980) proposed a simple, direct, and rapid method for the measurement of phytate in purified HCl grain extracts without additional digestion steps used in previous methods. The assay is based on the reaction between ferric chloride and sulfosalicylic acid and involves the "Wade reagent" originally used for the detection of phosphate esters separated by paper chromatography (Latta and Eskin, 1980). The Wade

reagent is simply a mixture of 0.03% ferric chloride (FeCl3·6H20), 0.3% sulfosalicylic acid, and distilled water and is pink in color due to the reaction between ferric chloride and sulfosalicylic acid (Latta and Eskin, 1980). The resulting compound absorbs maximally at a light wavelength of 500 nm. When phytate is present, phosphate esters bind to the ferric chloride causing it to be unavailable to the sulfosalicylic acid and thus resulting in decreased color intensity (Latta and Eskin, 1980). Latta and Eskin (1980) provided evidence for the efficacy of their method in predicting phytate concentrations by obtaining a coefficient of determination value of 1.0 when standard phytate concentrations were plotted against absorbance values. Furthuremore, comparisons of phytate measurements in grain samples between the method described above and a standard method involving the use of digestion steps showed that the two methods ranked samples identically and were comparable with respect to the phytate concentrations measured in each sample (Latta and Eskin, 1980).

Vaintraub and Lapteva (1988) showed that the methods of Latta and Eskin (1980) can be used on unpurified seed extracts, which would further reduce the time to determination. Crude and purified extracts from soybean meal, pea meal, and sunflower isolate were assayed for phytate; pairwise *t*-tests of the results indicated that there was no difference between the crude and purified extracts for any of the paired samples analyzed. Furthermore, Vaintraub and Lapteva (1988) showed that the standard deviation for the purified extracts was greater and the sensitivity was decreased due to extract dilution compared to the crude extracts. They concluded that the sensitivity of the crude extract assay is 15-fold higher than that of the original method using purified extracts. Therefore, using crude extracts rather than purified extracts can reduce time and increase precision and sensitivity without negatively affecting the results (Vaintraub and Lapteva, 1988). However, contrasting results

by Fruhbeck et al. (1995) suggested that purified extracts should be used for more reliable results.

High-performance liquid chromatography (HPLC) procedures for phytic acid measurement have been developed that produce very accurate results and are able to separate the phytic acid from lower level polyphosphates (Talamond et al., 2000). However, their use requires specialized and expensive equipment (Fruhbeck et al., 1995; Vaintraub and Lapteva, 1988).

Method of Available Phosphorus Measurement

The available P measurements described by Raboy et al.(2000) and Shi et al.(2000) are derived from the classic total P assay from Chen et al.(1956). In Chen et al. (1956), total P is measured by wet-ashing biological material and combining the digested P with ammonium molybdate, sulfuric acid, ascorbic acid, and distilled water. Upon reaction, a blue color develops and intensity is measured with 800 nm light in a spectrophotometer. Measurement of available P, which is of interest to breeders striving to improve P nutritional profiles, follows the same procedure except samples are extracted with mild HCl and the liberated P is assumed available for digestion. Such an approach is used for the qualitative identification of low-phytic acid mutants (Raboy et al., 2000). Segregants homozygous for the mutant alleles develop a deep blue color indicating a drastic increase in available P compared to wild type cultivars. Wild type and low-phytic acid lines do not differ in total P quantities (Raboy et al., 2000), which suggests that a quantitative measurement of available P using the above method with HCl extraction should be valid.

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CHAPTER 3: DEVELOPMENT OF QUANTITATIVE ASSAYS FOR PHYTATE AND AVAILABLE PHOSPHORUS FOR USE IN MAIZE BREEDING PROGRAMS

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ABSTRACT

Phytate is the dominant storage form of phosphorus (P) in mature cereal and oil grains. Phosphorus bound in phytate is unavailable to monogastric animals and thus contributes to water pollution through excretion in the manure. Also, phytate can exacerbate human mineral deficiencies. A rapid, inexpensive method of measuring phytate and available P levels in maize was derived from previously published assays and used to screen 50 inbred lines. Lab data was obtained from measurements taken in 96-well plates replicated and randomized in a RCB design. Measurements were also taken on grain yield, total P, protein, oil, methionine, lysine, tryptophan, and kernel weight. Field repeatability values for phytate and available P (0.78 and 0.91, respectively) suggest that these traits can be measured with adequate precision using the derived protocols. Correlations among all traits were calculated; the positive phytate:protein correlation commonly reported was also detected by our methods. A relationship between phytate and kernel weight indicates that breeding for low phytate may simply increase endosperm and kernel size. A detailed description of the phytate and available P protocol is provided as a reference for breeders interested in these traits.

INTRODUCTION

In mature seeds, typically 65 to 80% of total P is in the form of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakiphosphate), which can make up one to several percent of the seed dry weight (reviewed in Raboy, 2002). Nearly all of the phytic acid found in mature seeds is found bound to minerals such as K^{+}, Mg^{++}, Ca^{+}, Zn^{+}, Ba^{++}, and Fe^{+++} to form a mixed cation salt known as phytate (reviewed in Lott et al., 2000). Inorganic P and cellular P (a component of cellular membranes, DNA, RNA, etc.) are other forms of P in seeds and are generally referred to as "available P" (Raboy, 2001).

Lott et al. (2000) estimated that 65% of the elemental P sold and applied as fertilizer worldwide is sequestered into harvested crop seeds and fruits as phytic acid. Cereal grains and oil seeds have particularly high concentrations of phytate (O'Dell, 1972), which is known to negatively impact the environment in addition to human and animal nutrition. Because monogastric animals such as swine and poultry lack the enzyme phytase, most of the P in their maize based diets passes into the feces and onto the landscape where it contributes to surface water eutrophication (Sharpley et al., 1994). For example, the bioavailability of P in maize is less than 15% (Cromwell and Coffey, 1991). Also, phytate is a strong chelator of important minerals such as iron and zinc (Erdman, 1981) and therefore contributes to human micronutrient deficiencies in developing countries (Raboy, 2002). Over two billion people worldwide suffer from micronutrient deficiencies (Graham and Welch, 1996).

Many of the problems associated with P in maize grain are not due to the concentration of total P per se, but rather to the fact that most of the P is bound in phytate (Raboy, 2001). Therefore, it would be desirable to increase the amount of available P (P_A)

and reduce the amount of phytate in maize grain. Low phytic acid (*lpa*) mutant lines that have greatly reduced levels of phytate and concomitant increases in P_A have been developed in a variety of crop species, including maize, and have provided valuable information regarding the biochemical, physiological, and nutritional roles of phytate (Raboy et al., 2001; Raboy, 2002). However, slight yield reductions among these *lpa* lines have been reported in maize (Ertl et al., 1998) and germination problems have been observed in other crops (Hulke et al., 2004). Another approach would be to develop a selection program designed to decrease phytate and increase P_A while maintaining or improving other traits important to maize production. The success of modification through selection depends upon the ability to differentiate germplasm and lines by the measurement of traits in a high-throughput, inexpensive manner that can be incorporated into normal breeding operations. Furthermore, because repeatability sets an upper limit to heritability (Falconer and Mackay, 1996), precision of phytate and P_A measurements should compare with other traits commonly measured. Methods have been developed for the measurement of phytate in plant material, grain samples, and feedstuffs, but most are expensive or time consuming requiring heating, cooling, and multiple rounds of centrifuging (Vaintraub and Lapteva, 1988). Available P assays have been used to qualitatively identify *lpa* mutants, but quantitative determinations of P_A among maize lines have not been reported. It was our objective to design and report an assay coupled with careful experimental design and analysis for the simple, relative, and quantitative measurement of phytate and P_A in ground maize samples from the field. We have used this protocol to estimate phytate and P_A concentrations among 50 inbred lines grown in the summer of 2004. Also, measurements were made on yield, kernel weight, protein, oil, lysine (K), tryptophan (W), methionine (M), and total P. The genetic coefficient

of variation (CV_G) and repeatability for each trait in addition to phenotypic correlations between traits were calculated. This study was designed to provide insight into the possibility of measuring phytate and P_A for modification via a selection program, provide estimates of phenotypic correlations that may be important during selection, and determine differences between inbred backgrounds with respect to the traits of interest. A detailed description of the experimental design, lab protocol, and analysis for phytate and P_A will be given as a reference for plant breeders.

MATERIALS AND METHODS

Genetic Material

A set of 50 inbred lines representing the Iowa lines from B73 to B129, four North Carolina lines, one Nebraska line, Mo17, and 10 unreleased lines were included in this experiment and are presented in Table 1. Twenty entries in this group represent Stiff Stalk (SS) germplasm and 30 entries represent Non-Stiff Stalk (NSS) germplasm.

Field Design and Sampling

The 50 lines were evaluated in a randomized complete block design (RCBD) with two replications at Ames, IA during the summer of 2004. Plots consisted of two rows 5.49 m long with 0.76 m between rows. The plots were overplanted and thinned to a uniform plant density of 62 140 plants ha^{-1}. The lines were allowed to open pollinate and both rows were hand harvested for yield estimation and sampling. An approximately 454 g grain sample was taken from each field plot for quality analysis and a subsample consisting of 30 whole kernels was taken from each field plot sample and milled so that 70% of the millings passed through an 80 micron mesh screen.

Traits

Yield was estimated by hand harvesting both rows, drying the ear grain down to 15% moisture, weighing the shelled grain, and converting to Mg ha^{-1}. Protein and oil contents were predicted by near-infrared reflectance (NIR) of whole grain. Kernel weight was estimated as the number of grams per 250 whole kernels. Quantification of total P in the ground subsamples was conducted at the Iowa State University Soil and Plant Analysis Laboratory using EPA method 3051 (EPA, 2003). Total P concentrations are reported in parts per million (ppm). Amino acids were quantified using a microbial assay (Scott et al., 2004). Ten mg of grain was extracted and hydrolyzed in a well of a 96-well plate with 0.2 mg mL^{-1} Pepsin in 200 *mM* KCL pH 2.0. This extract was then used to supplement M9 minimal media (Sambrook and Russel, 2001) inoculated with a strain of *E.coli* auxotrophic for the amino acid of interest. For methionine, strain P4X (Jacob and Wollman, 1961) was used, for tryptophan strain CAG18455 (Singer et al., 1989) was used, and for lysine strain KL224 (Birge and Low, 1974) was used. Bacteria were grown until the culture reached a plateau in cell density, which was quantified by measuring the scattering of 595nm light. Standards of pure amino acids were used to verify that the amount of extract used fell into the linear range of the assay and to convert optical density values into amino acid concentrations. Each sample was measured in triplicate.

Measurement of Phytate and P_A

The colorimetric assays of Vaintraub and Lapteva (1988) and Raboy et al. (2000) were modified and used together with careful experimental design and analysis to quantify relative phytate and P_A concentrations per sample, respectively. Three lab replications per field block were used where each lab replication contained all 50 plots in a field block along

with 10 standards on a 96-well plate randomized in a row-column lattice design. The 10 standard concentrations are presented in Table 2. Ten mg (± 0.2 mg) of kernel tissue from each subsample were placed in assigned wells and 200 µL of 0.65 *M* HCl was added to each well. The 96-well plates were placed on a shaker at room temperature overnight (~12 hours) for extraction, removed, and centrifuged at 3000 RPM for 20 minutes.

Two separate evaluation plates were used to measure both phytate and P_A from each sample included on a single extraction plate. Thirty µL of extract was transferred to each evaluation plate with the integrity of the extraction plate randomization maintained. An equal volume of the P_A and phytate standards were placed in the appropriate wells. For the measurement of P_A, 130 µL of distilled deionized (dd) H_20 and 100 µL of P_A reagent were added to each well. The P_A reagent was made immediately before use and consists of: 2 parts dd H_2O, 1 part 0.02 *M* ammonium molybdate, 1 part 0.57 *M* ascorbic acid, and 1 part 3 *M* sulfuric acid. The P_A evaluation plates were allowed to sit for 15 to 20 minutes to develop a blue color. Optical density was then measured on a 96-well spectrophotometer at a wavelength of 820 nm. For the measurement of phytate, 200 µL of Wade reagent was added to each well and allowed to react for 15 minutes before optical density was measured at a 490 nm wavelength. The Wade reagent can be stored in a refrigerator for one month and consists of 2.5 g of 5-sulfosalycyclic acid, 0.25 g of $FeCl_3 \cdot 6H_20$, and 150 mL of dd H_20. The above solution was refrigerated overnight and adjusted to a pH of 3.05 with NaOH the following day. After pH adjustment, dd H_2O was added for a final volume of 200 mL.

Phytic acid dodecasodium salt from corn (PADDSS; Sigma P-8810) and KH_2PO_4 (Sigma P-5379) were used for the phytate and P_A standards respectively. Stock solutions for the standards were made by dissolving 10 mg of PADDSS per 1 mL of 0.65 *M* HCl and 0.11

g of KH_2PO_4 in 250 mL 0.65 *M* HCl. Table 2 presents the volumes of standard stock solution, HCl, and calculated concentration of each standard for phytate and P_A.

Statistical Analysis

The data set from this experiment consists of two levels, laboratory and field plot data, and adjustments for outliers were made at each level. The traits phytate, P_A, lysine, methionine, and tryptophan were first analyzed at the laboratory level and reanalyzed at the field plot level along with yield, protein, oil, kernel weight, and total P. For prediction of P_A, lysine, tryptophan, and methionine subsample concentrations, optical density (OD) least-squares means (LSmeans) were calculated with a mixed model for each field block where entry and plate were considered fixed and random respectively. A prediction equation was computed for each field block by the regression of known standard concentrations on their OD values. Prediction equations were then used to estimate concentrations for the above traits in each subsample on each plate. Predicted subsample concentrations were then reanalyzed with a mixed model identical to the model used for analysis of OD values. Concentration LSmeans were calculated for each line by field block combination (i.e. for each field plot). The same analysis was performed for phytate with the exception of fitting row and column nested within plate as random factors to account for the variation contributed by these factors regarding this trait. Phytate, P_A, lysine, tryptophan, and methionine field plot LSmeans were subsequently treated as single plot measurements.

To estimate the lab repeatability for these traits, a completely random model was fit and repeatability (r_L) was calculated as:

$$r_L = \sigma_L^2 / [\sigma_L^2 + (\sigma_e^2 / r)]$$

where r is the number of lab replications per field block ($r = 3$), σ_L^2 is line variance, and σ_e^2 is the error variance at the laboratory level.

Data on all traits in this experiment at the field plot level were analyzed as a RCBD with both line and replication fit as random effects. Field plot repeatability was estimated as:

$$r_F = \sigma_L^2 / [\sigma_L^2 + (\sigma_e^2 / r)]$$

where r is the number of field replications ($r = 2$). Variance components were estimated at the field plot level in this case. Entry means were used for the calculation of correlation coefficients between each trait. Contrasts between heterotic groups, SS and NSS, were made for each trait. A genetic coefficient of variation (CV_G) was computed for each trait by dividing the line standard deviation by the trait mean and taken as a percentage.

RESULTS AND DISCUSSION

The standard curves used to predict phytate and P_A in this study were highly linear with R^2 values greater than 0.95 and 0.99, respectively. The strong linear relationship between OD and standard concentration indicate that these standard curves can accurately predict the relative concentration of phytate and P_A in each sample. A non-significant Anderson-Darling statistic ($P > 0.10$) and examination of normal probability plots confirmed that the distributions of both phytate and P_A values did not significantly deviate from normality. Distributions for phytate, P_A, and total P are displayed in Figure 1. Line was a highly significant ($P < 0.01$) source of variation for all traits measured. One way to compare degree of variability across traits with different units of measurement is the CV_G. The CV_G for phytate is nearly one-fourth the CV_G of P_A, which could result from a less precise assay

for phytate or less phytate variability within this set of inbred lines. According to the CV_G values estimated, P_A and yield are highly variable compared to protein, oil, kernel weight, phytate, and total P in this set of inbred lines. An intermediate level of variability was found for the three amino acids (Table 3).

Repeatability was calculated for all lab (r_L) and field (r_F) traits as a measure of data quality (Table 3). Repeatabilities are unitless and can be used to compare the quality of data for individual traits. The r_L values indicate the reliability of trait measurements within a single field plot while the r_F values indicate the reliability of measurements taken from the same inbred line, but from different field plots. The high r_F value for P_A suggests that measurements of this trait are as precise as other traits commonly measured in maize such as yield. Also, the r_L values calculated for P_A were higher than any of the other traits measured in the lab. The lower r_L and r_F values calculated for phytate suggest that either refinement of the phytate assay is needed, or more variability for this trait should be found. Nevertheless, the r_F of phytate (0.78) still suggests that this protocol can measure this trait with an adequate degree of reliability. For example, the r_F of phytate is substantially larger than that of total P (0.48) measured by a standard EPA method in a laboratory that specializes in analytical techniques.

The means for all fifty inbred lines included in this study are presented in Table 1 along with the SS, NSS, and experiment means. Because this study only included one environment, inference space for the relative performance of these inbred lines is limited. However, several previous authors investigating traits such as phytate and total P traits reported either nonsignificant genotype x environment interactions or little to no rank change among genotypes across environments (Miller et al., 1980; Raboy et al., 1984; Wardyn and

Russell, 2004). Therefore, we believe the measurements of these grain quality traits in one environment should, at least, reliably separate superior and inferior lines. The best entry with regard to P_A was B90 with a P_A concentration 5.34 μg mL^{-1} greater than the experiment mean. B90 proved to be an outstanding line regarding grain quality as it ranked third in protein content (114 g kg^{-1}), first in tryptophan concentration, and contained greater than average levels of methionine and lysine. However, B90 ranked 49th among the 50 inbred lines with respect to yield. B104 was determined to have the lowest phytate concentration (194.2 μg mL^{-1}), but lower than average protein, tryptophan, lysine, and methionine. Because the lines included in this experiment include a nearly equal number from SS and NSS genetic backgrounds, differences between these sources with respect to any of the traits evaluated can be determined and be of relevance to a breeder wanting to design a program around specific traits. Significant differences were found for yield, P_A, protein, and total P where the SS heterotic group was higher yielding. Higher values of P_A, protein, and total P were found among the NSS lines.

Correlation coefficients among the traits are presented in Table 4 and were calculated for two reasons: 1) to identify any trait relationships that should be considered during the process of improving lines or germplasm for phytate or P_A, and 2) for comparison with previously reported correlations to provide evidence for the efficacy of our protocol in measuring real phytate and P_A concentrations. The absence of a correlation between phytate and P_A is favorable with the goal of modifying both traits in consideration. However, the positive correlation found between phytate and protein is undesirable for the breeding of a cultivar with superior nutritional properties. The positive relationship between phytate and protein (r = 0.51) detected in the present study is consistent with a secondary selection

response reported by Raboy et al. (1989) upon analysis of the Illinois low and high protein lines in their 83rd generation of divergent selection. Also, Raboy et al. (1984) found a positive phytate:protein correlation among soybean and *G. soja* lines. Phytate levels in cultivated crops are also expected to be highly and positively correlated with total P (Raboy et al., 2001). The correlation between phytate measured with the protocol detailed in this study and total P measured by the Iowa State University Soil and Plant Analysis Laboratory is also significantly positive ($r = 0.43$), but lesser in magnitude than previously reported correlations ($r > 0.90$). A Phenotypic correlation is a function of the underlying genetic correlation and heritabilities of the traits (Falconer and Mackay, 1996). Due to the lower repeatability values estimated for both phytate and total P, the genetic correlation is expected to be considerably larger. A negative correlation between yield and phytate indicates that development of high yielding lines with lower phytate should be an attainable goal, but P_A was found to be negatively correlated with yield. A slight correlation between phytate and lysine ($r = 0.31$) indicates that lowering phytate via selection may decrease this limiting amino acid if attention is not given to this relationship.

An important relationship to consider before the initiation of long term selection aimed at decreasing whole kernel phytate is that between phytate and kernel size. Because we measured phytate concentration on ground whole kernels and ~90% of phytate is found within the germ (O'Dell et al., 1972), larger kernels with larger endosperms may have a diluted concentration of phytate. Raboy et al. (1989) also alluded to this fact when they investigated the relationship between high oil maize lines and phytate. They speculated that whole kernel phytate and total P concentrations may be contingent on seed size and seed organ ratio alterations through indirect selection. We found a significant, negative

phytate:kernel weight correlation ($r = -0.39$), which is in accordance with the hypothesis that as seed size increases, whole kernel phytate decreases. This is most likely due to a larger endosperm to germ ratio and thus an increased amount of endosperm flour in the ground subsamples. In addition to the negative correlation estimated, kernel weight was found to be a highly significant covariate ($P < 0.01$) on phytate concentration and a selection program aimed at decreasing phytate in whole kernel samples may simply result in lines with larger kernels (Figure 2).

In summary, this study indicates that repeatable measurements of P_A and phytate can be made in an inexpensive, high-throughput manner by breeders. Phytate measurements were made with enough precision to detect previously reported correlations, but refinement of the phytate assay is necessary to increase repeatability to the levels of P_A and yield. Dilution and pH buffering of the extracts may improve results (Vaintraub and Lapteva, 1988). Furthermore, the weighing of 10 mg of kernel tissue into individual wells is still too laborious for the purpose of screening large numbers of breeding lines. Possible ways of circumventing this step are currently being investigated. The phenotypic correlations detected suggest that a form of multiple trait selection should be employed when striving to develop lines with enhanced nutritional profiles and yielding abilities. Finally, this study not only sought out a rapid phytate and P_A assay, but combined these assays with the careful experimental design and analysis commonly used by field scientists to improve data quality. Such an approach should be utilized when striving for simple, rapid measurements of quality traits not typically measured by breeders.

Table 1. Line means for yield, available phosphorus (PA), phytate, total phosphorus (total P), methionine (M), lysine (K), tryptophan (W), protein, oil, and kernel weight. Bottom three rows are the stiff stalk, nonstiff stalk, and experiment means, respectively.

Line	Yield	P_A	Phytate	Total P	M	K	W	Protein	Oil	Kernel weight†
	Mg ha^{-1}	µg mL^{-1}	µg mL^{-1}	ppm	mM	mM	mM	g kg^{-1}	g kg^{-1}	g $250K^{-1}$
B73	5.82	2.88	265.1	2525	0.437	0.404	0.188	80.0	38.5	70.0
B84	4.60	3.35	275.3	2531	0.474	0.487	0.177	86.5	33.0	71.9
B90	2.57	12.07	268.6	2935	0.437	0.453	0.278	113.5	37.5	73.5
B91	3.81	5.91	264.4	2893	0.353	0.399	0.223	102.5	37.0	61.9
B97	5.25	10.66	274.4	2709	0.291	0.413	0.184	92.0	36.0	82.2
B99	3.54	7.73	280.0	2906	0.361	0.350	0.190	102.5	33.5	61.7
B100	4.36	9.47	280.4	3031	0.414	0.474	0.206	95.0	38.5	65.9
B101	3.30	2.65	274.8	2756	0.552	0.735	0.223	101.5	38.0	56.0
B102	4.41	8.65	284.5	2769	0.403	0.404	0.187	96.0	39.0	67.5
B103	3.49	5.74	273.3	2983	0.465	0.562	0.199	96.0	38.0	71.6
B104	6.17	9.72	194.2	2995	0.380	0.296	0.184	78.0	34.0	79.9
B105	5.44	2.16	300.8	2784	0.431	0.588	0.198	95.0	46.0	56.2
B106	6.30	7.03	287.6	3036	0.289	0.476	0.169	91.5	36.5	60.5
B107	3.25	6.32	275.4	2960	0.377	0.513	0.261	91.0	37.0	72.4
B108	5.22	6.68	290.0	3116	0.390	0.342	0.182	100.0	35.5	60.8
B109	6.71	3.83	261.1	2955	0.498	0.419	0.186	87.0	38.0	68.4
B110	6.52	3.86	226.7	2401	0.359	0.386	0.185	85.4	35.5	67.2
B111	5.76	2.69	282.0	2614	0.419	0.424	0.208	84.5	36.5	64.5
B112	5.52	8.62	234.1	2420	0.361	0.548	0.190	81.5	35.0	65.6
B113	4.25	8.85	264.8	2664	0.403	0.585	0.156	98.0	34.5	65.4
B114	5.33	8.16	273.9	2860	0.394	0.549	0.256	87.0	37.0	73.8
B115	2.87	10.04	263.1	2906	0.438	0.478	0.226	108.5	43.0	76.8
B116	7.71	11.38	255.9	2886	0.336	0.531	0.183	99.0	35.0	81.1
B117	4.90	7.52	238.5	2685	0.364	0.328	0.174	105.5	35.5	77.7
B118	4.90	8.65	278.7	2707	0.319	0.365	0.185	102.5	37.0	78.4
B119	5.66	2.50	274.9	2776	0.287	0.445	0.233	72.6	37.1	70.5

B120	4.84	5.94	262.1	2536	0.468	0.380	0.214	93.5	34.5	66.8
B121	7.44	4.39	236.7	2488	0.388	0.288	0.168	78.5	36.0	71.2
B122	5.41	10.56	228.5	2568	0.400	0.487	0.186	93.5	36.0	65.9
B123	3.54	10.03	331.9	2593	0.418	0.451	0.200	109.0	39.0	43.2
B124	5.03	9.89	263.9	3282	0.429	0.368	0.169	102.6	36.0	59.0
B125	3.63	7.79	278.5	2892	0.502	0.373	0.183	97.0	35.5	65.4
B126	3.90	9.62	304.9	2885	0.449	0.608	0.162	118.5	31.5	73.2
B127	6.01	5.10	245.1	2542	0.393	0.425	0.195	87.5	32.5	73.6
NC394	1.43	9.79	275.1	2850	0.342	0.411	0.199	93.5	38.5	60.4
B129	6.01	7.19	247.4	2712	0.393	0.487	0.171	89.5	34.5	79.8
N196	3.65	6.07	222.4	2602	0.452	0.439	0.197	91.0	38.5	67.0
Mo17	5.44	6.20	267.1	2766	0.219	0.592	0.205	96.5	35.0	79.9
B73/B89‡	6.06	6.15	238.2	2384	0.428	0.336	0.176	78.5	38.5	74.0
BS31(R)C0‡	4.71	5.02	285.8	3022	0.475	0.350	0.169	96.5	36.0	55.7
BSKRL1(HI)C1‡	4.95	6.52	259.1	2580	0.380	0.412	0.144	81.4	32.9	79.9
BSKRL1(HI)C1‡	7.41	3.80	231.0	2299	0.309	0.447	0.194	70.6	36.1	67.9
BS32(R)C0‡	3.57	6.51	255.0	2960	0.362	0.428	0.164	99.0	46.0	72.2
BSKRL2(H1)C1‡	4.52	5.73	272.0	3077	0.374	0.384	0.195	102.5	36.0	47.6
NC386	4.82	4.62	256.2	2701	0.339	0.305	0.162	78.5	42.0	64.1
B97/B99‡	5.87	9.00	227.3	2584	0.328	0.385	0.169	89.5	36.0	67.3
BS13(S)C8‡	9.14	4.87	239.9	2380	0.325	0.325	0.213	70.0	38.0	65.6
NC376	2.84	6.65	294.0	3105	0.404	0.419	0.182	89.5	34.0	71.6
BSCB1(R)C12‡	3.54	4.35	274.5	3126	0.512	0.442	0.199	116.0	36.0	65.6
NC432	7.12	3.60	238.1	2593	0.462	0.366	0.177	75.5	38.0	65.2
SS §	**5.57***	**4.90****	**258.0**	**2671***	**0.416**	**0.440**	**0.189**	**86****	**36.0**	**68.9**
NSS §	**4.55***	**7.94****	**266.9**	**2827***	**0.383**	**0.434**	**0.196**	**97****	**37.0**	**67.5**
Mean	**4.98**	**6.73**	**263.5**	**2767**	**0.396**	**0.437**	**0.193**	**93.0**	**37.0**	**68.1**

*, ** Significant at the 0.05 and 0.01 probability levels, respectively.

† Kernel weight units are grams per 250 whole kernels.

‡ Uncoded inbred lines.

§ Traits that exhibited significant differences between genetic background means are indicated.

Table 2. Constitution of standards used for P_A and phytate prediction. The tabled values underneath the subheadings "Conc." were calculated from the phytate and P_A stock solution concentrations and quantity of stock in each standard. Values underneath the subheadings "Pred conc." are the predicted concentrations from the linear regression of optical density values on actual standard concentrations.

	P_A					Phytate				
Standard	HCl	Stock	Conc.	Pred conc. FR1	Pred conc. FR2	HCl	Stock	Conc.	Pred conc. FR1	Pred conc. FR2
	——µL——			————————µg mL^{-1}————		——µL——			————————µg mL^{-1}————	
1	1000	0	0.0	0.00	0.00	1000	0	0.0	0.5	-1.3
2	980	20	2.0	1.95	1.99	990	10	45.7	51.3	79.8
3	970	30	3.0	2.97	3.02	980	20	91.3	90.4	98.2
4	960	40	4.0	3.99	3.90	970	30	137.0	115.0	123.7
5	950	50	5.0	5.07	5.17	965	35	160.0	172.9	122.9
6	940	60	6.0	5.98	6.01	960	40	182.0	199.3	185.3
7	920	80	8.0	8.16	8.03	955	45	205.0	204.4	211.4
8	910	90	9.0	8.89	8.80	950	50	228.0	229.6	224.5
9	900	100	10.0	10.02	9.96	945	55	251.0	241.6	243.8
10	880	120	12.0	11.94	12.12	940	60	275.0	270.1	286.9

Table 3. Estimated variance components (line = σ_L^2, error = σ_e^2), lab and field repeatabilities (r_L and r_F respectively), mean, minimum, and maximum values, and genetic coefficients of variation (CV_G) for each trait.

Trait	Unit	σ_L^2	σ_e^2	r_L‡		r_F	Mean	Min	Max	CV_G
				FR 1	FR 2					
Yield	Mg ha^{-1}	1.97	0.49			0.89	4.98	1.43	9.14	30.00
P_A	μg mL^{-1}	6.21	1.25	0.98	0.99	0.91	6.73	2.16	11.90	39.00
Phytate	μg mL^{-1}	448.4	257.1	0.87	0.89	0.78	263.5	194.2	331.9	9.40
Total P	ppm	23 834	49 738			0.49	2767	2299	3283	8.30
Methionine	mM	3.8×10^{-3}	0.9×10^{-3}	0.79	0.94	0.90	0.39	0.22	0.55	16.00
Lysine	mM	7.1×10^{-3}	2.3×10^{-3}	0.81	0.81	0.86	0.44	0.29	0.73	21.00
Tryptophan	mM	0.5×10^{-3}	0.4×10^{-3}	0.88	0.39	0.74	0.19	0.14	0.28	14.00
Protein	g kg^{-1}	126.20	5.24			0.98	92.6	70.0	118.5	12.00
Oil	g kg^{-1}	7.67	1.25			0.92	36.8	31.5	46.0	7.80
Kernel weight	g 250K^{-1}†	66.10	3.92			0.97	68.1	43.2	82.2	12.00

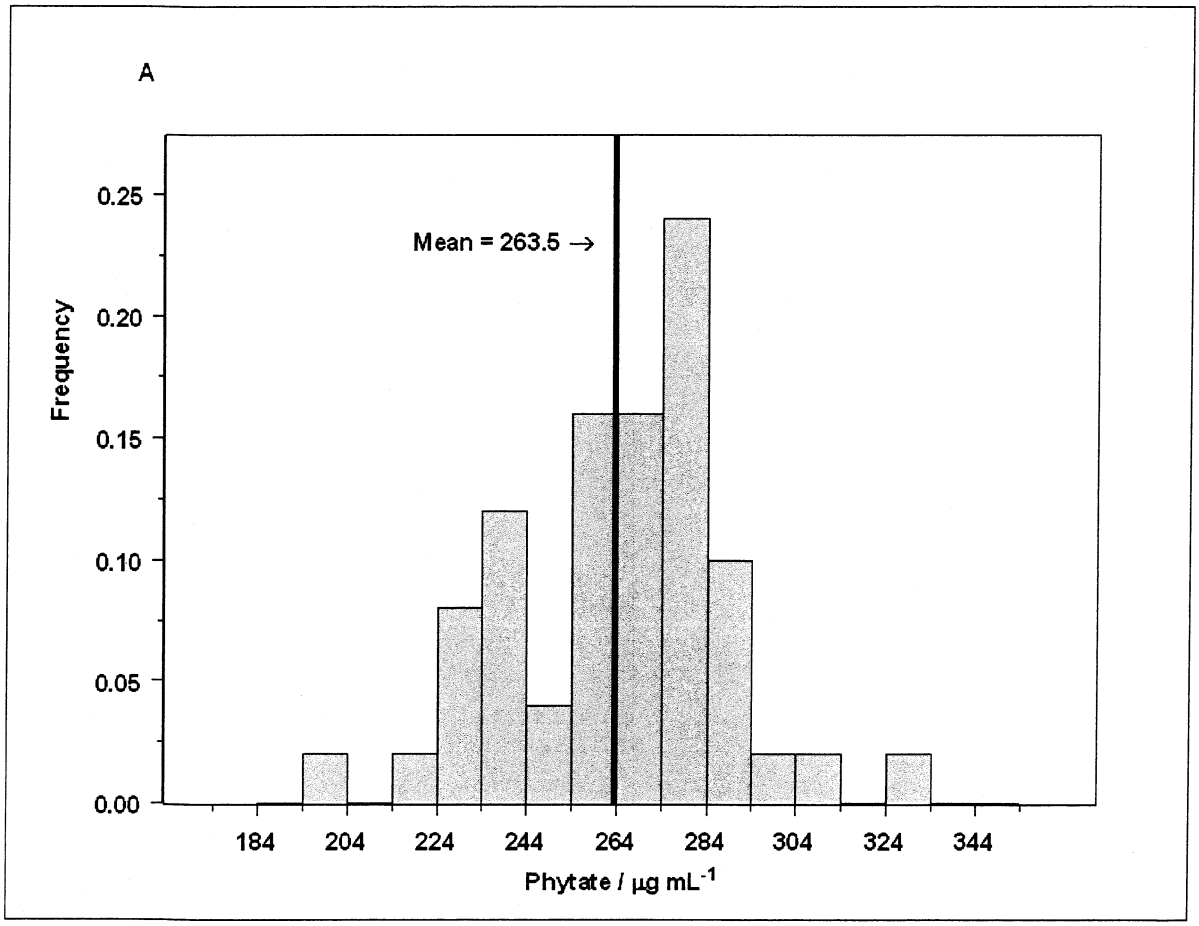
† Grams per 250 whole kernels.

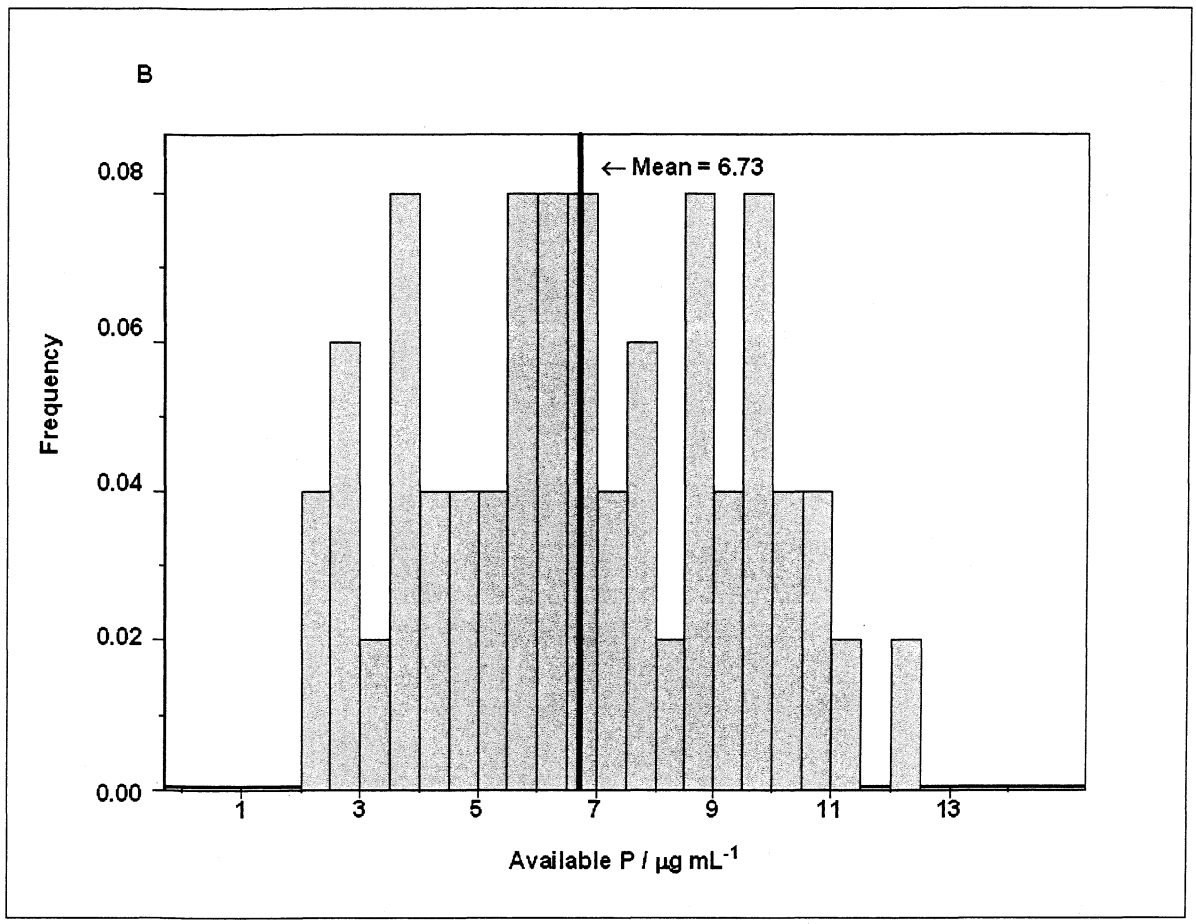
‡ Lab repeatability calculated for both field rep 1 and field rep 2.

Table 4. Simple correlation coefficients among traits of inbred lines grown in Ames during the summer of 2004.

Trait	Yield	P_A	Phytate	Total P	Methionine	Lysine	Tryptophan	Protein	Oil
	Mg ha^{-1}	—μg mL^{-1}—		ppm	——————mM——————			——g kg^{-1}——	
P_A	-0.29*								
Phytate	-0.47**	0.03							
Total P	-0.47**	0.27	0.43**						
Methionine	-0.31*	-0.19	0.17	0.16					
Lysine	-0.25	0.02	0.31*	0.07	0.16				
Tryptophan	-0.25	0.01	0.15	0.11	0.05	0.27			
Protein	-0.64**	0.46**	0.51**	0.56**	0.28	0.30*	0.12		
Oil	-0.15	-0.15	0.11	0.07	0.06	0.03	0.18	0.01	
Kernel weight †	0.19	0.21	-0.39**	-0.14	-0.27	0.00	-0.03	-0.17	-0.23

*, ** Significant at the 0.05 and 0.01 probability levels, respectively.
† Units are grams per 250 whole kernels.


A
Mean = 263.5 →
Frequency
0.25
0.20
0.15
0.10
0.05
0.00
184
204
224
244
264
284
304
324
344
Phytate / µg mL-1


B
← Mean = 6.73
0.08
0.06
0.04
0.02
0.00
Frequency
1
3
5
7
9
11
13
Available P / µg mL-1

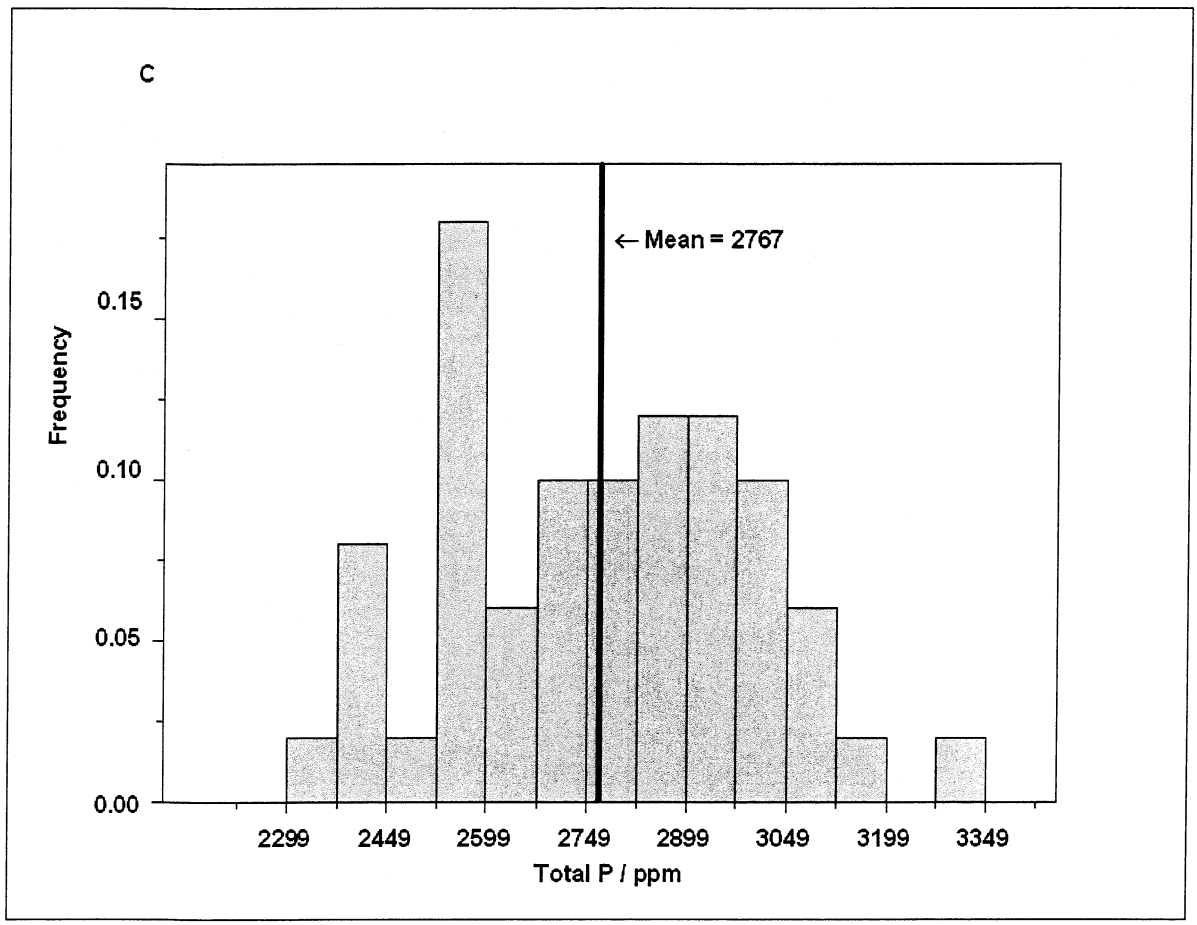


Fig. 1. Distributions of phytate (A), available P (B), and total P (C) for the 50 inbred lines evaluated. Reference lines indicating the experiment mean for each are shown.

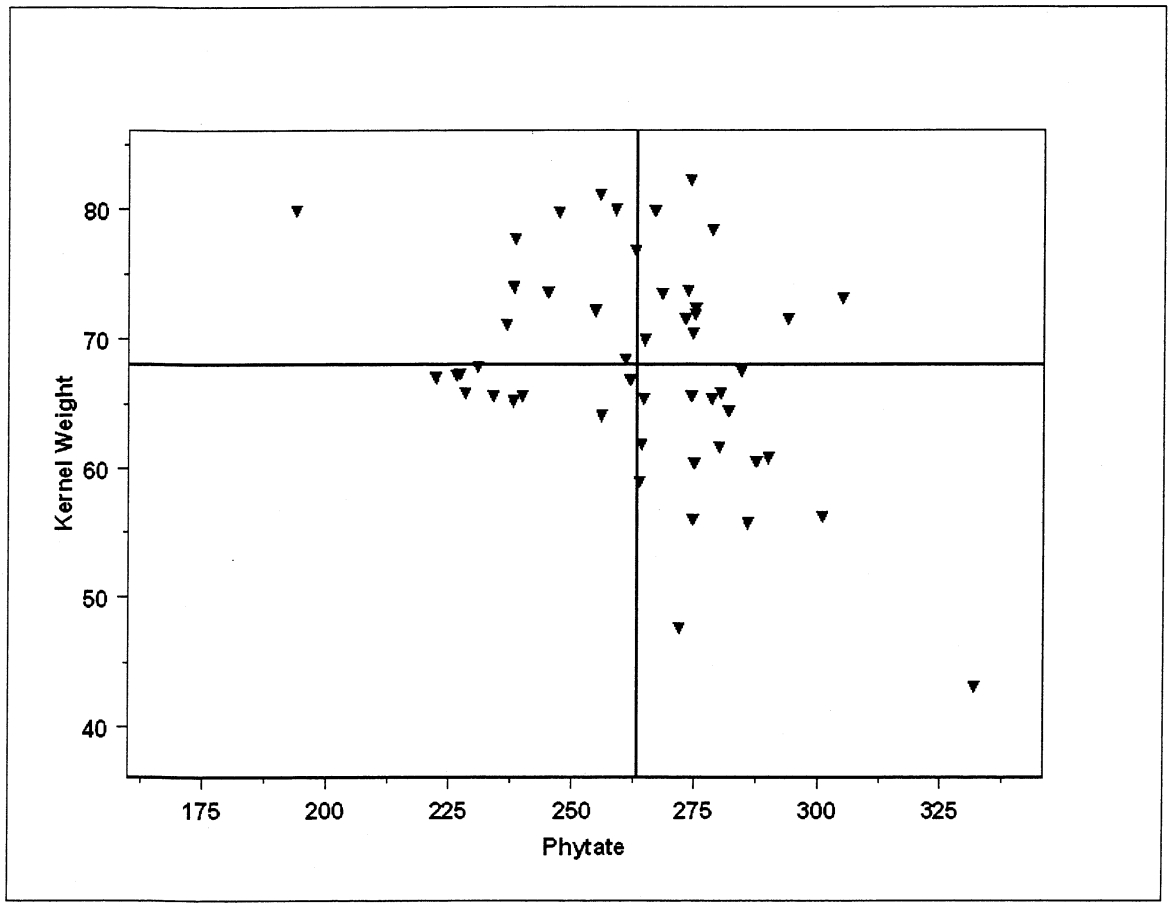


Fig. 2. Kernel weight (g per 250 kernels) versus phytate (μg mL^{-1}). Horizontal and vertical reference lines indicate the experiment means of kernel weight and phytate, respectively.

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CHAPTER 4: POTENTIAL OF PHYTATE AND AVAILABLE PHOSPHORUS IMPROVEMENT THROUGH SELECTION IN A MAIZE POPULATION

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ABSTRACT

Phytate is the dominant storage form of phosphorus (P) in maize grain. Because P bound in phytate is unavailable to monogastric livestock, it is excreted in the manure and contributes to surface water pollution. Furthermore, diets of swine and poultry are supplemented with phytase or phosphate to meet the animals' P requirements. Also, phytate can exacerbate human mineral deficiencies in developing nations. Therefore, it would be desirable to decrease phytate and increase available P in maize grain while improving other traits of agronomic importance. Broad-sense heritability (H^2) estimates for phytate, available P, and several other traits were obtained from a breeding population of S1 lines. Phytate and available P measurements were designed to be rapid, inexpensive, and easily incorporated into a breeding program. The results indicate that available P is highly heritable ($H^2 = 0.86$), but the lowest H^2 estimate among all traits was for phytate ($H^2 = 0.59$). These results suggest that refinement of the phytate assay is still needed to increase precision, but provide an optimistic start for the breeding of traits not typically measured. Construction of several selection indices and truncation-index combinations indicate that progress can be made for available P, phytate, yield, moisture, and root and stalk lodging in the desired directions. A significant, positive phytate:protein correlation (0.37 phenotypic; 0.51 genetic) was found in the present study and is accordance with several previous reports. Due to the phytate:protein

correlation and a significant, negative phytate:starch correlation (-0.33 phenotypic; -0.45 genetic), it is hypothesized that selection on phytate would alter the germ to endosperm ratio of the seed resulting in larger, starchier kernels. Evidence for this hypothesis is apparent among the selection methods investigated in which protein and kernel weight consistently decrease and increase, respectively.

INTRODUCTION

In mature seeds, typically 65 to 80% of total P is in the form of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakiphosphate), which can make up one to several percent of the seed dry weight (reviewed in Raboy, 1990). Most phytic acid of mature seeds is found bound to minerals such as K^{+}, Mg^{++}, Ca^{+}, Zn^{+}, Ba^{++}, and Fe^{+++} to form a mixed cation salt known as phytate (reviewed in Lott et al., 2000). Inorganic P and cellular P (a component of cellular membranes, DNA, RNA, etc.) are other forms of P in seeds and are generally referred to as "available P" (Raboy, 2001).

Cereal grains and oil seeds have particularly high concentrations of phytate (O'Dell, 1972), which is known to negatively impact the environment in addition to human and animal nutrition. Because monogastric animals such as swine and poultry lack the enzyme phytase, most of the P in their maize based diets passes into the feces and onto the landscape where it contributes to surface water eutrophication (Sharpley et al., 1994). For example, the bioavailability of P in maize diets is less than 15% (Cromwell and Coffey, 1991). Also, phytate is a strong chelator of important minerals such as iron and zinc (Erdman, 1981) and therefore contributes to human micronutrient deficiencies in developing countries (Raboy, 2002).

Many problems associated with P in maize grain are not due to the concentration of total P per se, but rather to the fact that most of the P is bound in phytate (Raboy, 2001). Therefore, it would be desirable to increase the amount of available P (P_A) and reduce the amount of phytate in maize grain. Low-phytic acid (*lpa*) mutant lines having normal levels of total P, but greatly reduced levels of phytate and concomitant increases in inorganic P have been developed in maize (Raboy et al., 2000). However, slight yield reductions among these *lpa* hybrids have been reported in maize (Ertl et al., 1998) and germination problems have been observed in other crops (Hulke et al., 2004). Another approach would be to develop a recurrent selection program designed to decrease phytate concentration and increase P_A levels while maintaining or improving other traits important to maize production.

Phytate and total P measurements in a variety of crops including wheat (Raboy et al., 1991), soybean (Raboy and Dickinson, 1993; Raboy et al., 1984), dry beans (Lolas and Markakis, 1975) and oat (reviewed in Maga, 1982) indicate that significant genetic variation exists for such traits. However, such information on phytate and P_A is limited in maize, especially for standard breeding populations. Raboy et al. (1989) found a 2.5-fold difference in phytic acid concentration between Illinois high and low protein lines in their 83rd generation of divergent selection. Based on this secondary response to selection, Raboy et al. (1989) concluded that phytic acid can be altered through selection, but correlations between phytic acid and protein may hinder the breeding of a cultivar with enhanced nutritional properties. Wardyn and Russell (2004) estimated the broad-sense heritability (H^2) of total P in a diverse population of S_1 lines to be 0.82 and concluded that total P should be easily modified by selection.

In addition to population genetic variation, the success of a breeding program is contingent upon the ability to measure the characters of interest in a rapid, inexpensive, and reliable manner that can be easily incorporated into normal operations. Most methods of measuring phytate are expensive, laborious, or require specialized equipment and therefore hinder investigation of this trait by breeders wishing to screen a large number of lines. To the knowledge of the authors, quantitative measurements of P_A in maize breeding populations have not been reported. It was the primary objective of this study to report H^2 estimates for phytate and P_A in grain samples obtained during machine harvest and measured with rapid protocols derived from the methods of Vaintraub and Lapteva (1988) and Raboy et al. (2000), respectively. Heritabilities of other characters typically quantified by maize breeders were also estimated for comparison. A second objective was to estimate phenotypic and genotypic correlations for the identification of potential undesirable secondary selection responses. Finally, selection differentials from a set of selection indices involving phytate, P_A, yield, grain moisture, and root and stalk lodging were developed to investigate the possibility of modifying phytate and P_A along with agronomic traits in a breeding program.

MATERIALS AND METHODS

Germplasm, Field Design, and Evaluation

Plants from the BS31 population were randomly self-pollinated to produce 90 S_1 families. These 90 families were evaluated in a randomized complete block design (RCBD) with two replications each at two Iowa locations (Ames and Carroll) during the summer of 2004. Plots consisted of two rows 5.49 m long with 0.76 m between rows. The plots were overplanted by machine and thinned to a uniform plant density of 62 140 plants ha^{-1}. The

lines were allowed to open pollinate and an approximately 454 g grain sample was obtained from each plot by an automated sampler during machine harvest. A subsample consisting of 30 whole kernels was taken from each field plot sample and milled so that 70% of the millings passed through an 80 micron mesh screen.

Data collected on plots were machine-harvestable grain yield (Mg ha^{-1}) adjusted to 155 g kg^{-1} grain moisture at harvest, grain moisture (g kg^{-1}), root lodging (percentage of plants leaning more than 30° from vertical), stalk lodging (percentage of plants broken at or below the primary ear node), test weight (kg m^{-3}), and silk emergence (days after planting in which 50% of the plants had emerged silks in Ames). Data collected on field plot samples included kernel weight (g per 250 whole kernels) and protein, oil, and starch (g kg^{-1}; all predicted with near infrared reflectance spectroscopy of whole grain).

Measurement of Phytate and Available Phosphorus

The colorimetric assays of Vaintraub and Lapteva (1988) and Raboy et al.(2000) were modified and used in conjunction with careful experimental design and analysis to quantify relative phytate and P_A concentrations per sample, respectively. A 96-well plate containing all 90 plots in a field block along with six standards constituted a lab block. Three lab blocks were nested within each field block and were randomized in a row-column alpha lattice design. The six standard concentrations are presented in Table 1. Ten mg (± 0.2 mg) of millings from each subsample were placed in assigned wells and 200 μL of HCl (0.65 *M*) was added to each well. The plates were placed on a shaker overnight at room temperature (~12 hours) for extraction, removed, and centrifuged at 3000 RPM for 20 minutes.

Measurements of both phytate and P_A were obtained from the same samples contained in a single lab block by nesting two evaluation plates within each lab block. Thirty

μL of supernatant were transferred to each evaluation plate with the integrity of the lab block randomization maintained. Thirty μL of the phytate and P_A standards were placed in assigned wells of each plate to form phytate and P_A evaluation plates. For the measurement of P_A, 130 μL of distilled deionized (dd) H_2O and 100 μL of P_A reagent were added to each well of the P_A evaluation plates. The P_A reagent was made immediately before use and consists of: 2 parts dd H_2O, 1 part 0.02 *M* ammonium molybdate, 1 part 0.57 *M* ascorbic acid, and 1 part 3 *M* sulfuric acid (Chen et al., 1956). The P_A evaluation plates were allowed to sit for 15 to 20 minutes to develop a blue color. Optical density (OD) was then measured with a 96-well spectrophotometer at a wavelength of 820 nm. For the measurement of phytate, 200 μL of Wade reagent was added to each well of the phytate evaluation plates. Reaction was allowed to take place for 15 minutes before OD was measured with 490 nm light. The Wade reagent can be stored in a refrigerator for one month and consists of 5-sulfosalycylic acid (2.5 g), $FeCl_3 \cdot 6H_20$ (0.025 g), and dd H_20 (150 mL). The above solution was refrigerated overnight and adjusted to a pH of 3.05 with NaOH the following day. After pH adjustment, dd H_2O was added for a final volume of 200 mL.

Phytic acid dodecasodium salt from corn (PADDSS; Sigma P-8810) and KH_2PO_4 (Sigma P-5379) were used for the phytate and P_A standards respectively. Stock solutions for the standards were made by dissolving 0.01 g of PADDSS per 1 mL of 0.65 *M* HCl and 0.11 g of KH_2PO_4 in 250 mL 0.65 *M* HCl. Table 1 presents the volumes of standard stock solution, HCl, and calculated concentrations of each standard for phytate and P_A.

Statistical Analysis

The data set from this experiment consists of two levels, laboratory (phytate and P_A) and field plot data, and adjustments for outliers were made at each level. The traits phytate

and P_A were first analyzed at the laboratory level in which least-squares means (LSmeans) for each field plot were obtained and used for analysis at the field level with the other traits measured.

Prediction equations for P_A and phytate were developed for each field block by the regression of known standard concentrations on their OD values. The coefficients of determination obtained upon this regression were greater than 0.99 across both traits and the four field blocks. The standard curves were used to predict phytate and P_A concentration in each subsample of each lab block. Predicted concentrations were then analyzed for each field block separately with a mixed model where family was considered fixed and lab block, row, and column were all considered random factors. Row and column were both nested within lab block. Least-squares means were obtained for each family by field block combination (field plot). Phytate and P_A field plot LSmeans were subsequently treated as single field plot measurements.

For analysis at the field level, all factors were fit as random factors in PROC MIXED (SAS Institute, 2003a) to estimate variance components for family, family by environment, and error (σ_F^2, σ_{FE}^2, and σ_e^2 respectively). The Wald Z-test was used to test if the variance components were greater than zero (SAS Institute, 2003b) with the exception of σ_{FE}^2 which was tested by solving expected mean squares and calculating an F-value. Broad-sense heritability estimates on a family mean basis for each trait were calculated with

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + (\sigma_{GE}^2 / e) + (\sigma_e^2 / re)}$$

where e is the number of environments ($e = 2$) and r is the number of replications (field blocks per environment; $r = 2$). Entry means across environments were used in the

calculation of phenotypic correlations. Genotypic correlations were calculated by equating observed mean cross products with their expected values and solving according to Bernardo (2002).

Estimated selection differentials based on a 10% selection intensity were calculated from a series of indices involving agronomic traits (yield, grain moisture, root lodging, and stalk lodging) and P traits (phytate and P_A). To avoid problems of scale, entry LSmeans were transformed by $(X_i - \overline{X})/\sigma_i$, where X_i, $\overline{X}$, and σ_i are the entry mean, experiment mean, and standard deviation of trait *i*. The heritability index and rank summation index from Smith et al. (1981) and Mulamba and Mock (1978) were used in various forms which are individually described here.

Heritability index 1: Transformed family means were ranked according to the index: $I = H^2_{yield}(X_{yield}) - H^2_{moist}(X_{moist}) - H^2_{RL}(X_{RL}) - H^2_{SL}(X_{SL})$ where the subscripts *RL* and *SL* stand for root lodging and stalk lodging, respectively. The top 30 families (33%) were truncated and re-ranked based on $I = H^2_{P_A}(X_{P_A}) - H^2_{phytate}(X_{phytate})$. The top nine families (10%) were selected and "selected means" were calculated for each trait.

Heritability index 2: Transformed family means were ranked according to the index:

$$I = H^2_{yield}(X_{yield}) - H^2_{moist}(X_{moist}) - H^2_{RL}(X_{RL}) - H^2_{SL}(X_{SL}) + H^2_{P_A}(X_{P_A}) - H^2_{phytate}(X_{phytate})$$

and the top nine families were selected.

Rank summation index 1: Families were ranked in descending order for yield and ascending order for moisture, root lodging, and stalk lodging. The four rank values were summed and the top 30 families were truncated. Rank values were given to the truncated

families based on ascending and descending rank orders for phytate and P_A respectively and the top nine families were selected.

Rank summation index 2: Rank values for yield, moisture, root lodging, stalk lodging, phytate, and P_A were given and the top nine families were selected based on the sum.

Method 5: Data on yield and moisture were converted to quintals per hectare and percent respectively. Families were assigned two rank values; one based on the index $I = H^2_{yield}(X_{yield}) - H^2_{moist}(X_{moist}) - H^2_{RL}(X_{RL}) - H^2_{SL}(X_{SL})$ and the other based on $I = H^2_{P_A}(X_{P_A}) - H^2_{phytate}(X_{phytate})$. The top nine entries were selected on the sum of the two ranks.

Method 6: A heritability index like those above was used on the transformed entry LSmeans for the traits yield, moisture, phytate, and P_A. Selections (top 10%) were made based on the index value.

Method 7: A heritability index was used on the transformed entry means for the traits yield, phytate, and P_A.

Selection differentials for all traits were also calculated for selections made on a heritability index involving only phytate and P_A, selections on phytate alone, and selections on P_A alone.

RESULTS AND DISCUSSION

Family was a highly significant ($P < 0.01$) source of variation for all traits while family by environment effects were significant for yield, moisture, root and stalk lodging, protein, and starch. No significant differences between the means of the locations were

found for any trait studied. The data sets for phytate and P_A were determined to be normal by inspection of normal probability plots and calculation of non-significant ($P > 0.05$) Anderson-Darling statistics. Family effects for P_A were the largest source of variation with σ^2_{FE} and σ^2_e small in comparison. The experiment mean for P_A was 4.20 μg mL^{-1} and the range of entry means was 2.44 to 8.08 μg mL^{-1} (Table 2). Although the variance attributed to family effects was highly significant with respect to phytate, σ^2_e was the largest the source of variation indicating that the assay used for phytate determination should be refined to improve precision. The experiment mean for phytate concentration was 181.4 μg mL^{-1} and the range was 157.5 to 200.6 μg mL^{-1}. Various results regarding σ^2_{FE} effects for phytate and similar traits such as total P have been reported. The lack of a family by environment interaction for both phytate and P_A agrees with results reported in soybean by Raboy and Dickinson (1993). In contrast, Wardyn and Russell (2004) found highly significant σ^2_{FE} for total P among a population on S1 lines, but reported the σ^2_{FE} to be only 18% of the σ^2_F and that selection differentials based on data from one and two environments were only slightly different (5%).

Broad-sense heritability (H^2) estimates range from 0.59 to 0.94 across the traits studied (Table 2). The H^2 for each trait simply reflects the degree of genetic determination and can be used for comparisons of genetic diversity, environmental influences, and error across traits differing in unit and scale. Notably, the H^2 of P_A was 0.86 and was higher than that of yield and root and stalk lodging, which are all traits commonly improved through selection practices. The method of P_A determination described above is typically used as a qualitative identifier of lines carrying the *lpa* mutation (Raboy et al., 2000). The large H^2

found for P_A suggests that this laboratory method also can be used for quantitative determination of P_A and that modification via selection could be made. On the other hand, the lowest H^2 estimated was that for phytate (0.59). The high error generated by the experimental, rapid phytate assay used is the probable cause of this low H^2 estimate rather than a lack of genetic variation.

Phenotypic correlations between P_A, phytate, and all other traits were calculated to identify any traits that may be impacted by selection on phytate and P_A (Table 3). No significant correlations were found between P_A and any other character. However, significant phenotypic correlations were found between phytate and protein and phytate and starch (0.37 and -0.33 respectively); genetic correlations were slightly larger in magnitude (0.51 and -0.45 respectively). Positive phytate-protein correlations have been reported in wheat (Raboy et al., 1993) and soybean (Raboy et al., 1984) in addition to simultaneous selection responses between these two traits in maize (Raboy et al., 1989). Because only trace amounts of phytate are found in the endosperm of mature maize kernels (O'Dell et al., 1972), the negative correlation estimated between starch and phytate was expected. These results indicate that selection aimed at lowering phytate levels may alter the endosperm to germ ratio. Although no significant correlation was found between phytate and kernel weight in this study, a previous screening of established inbred lines suggested otherwise with a significant, negative correlation of -0.39 (Lorenz, 2005). Despite common reports of high correlations between phytate and total P (reviewed in Raboy et al., 2001), our findings suggest that no correlation exists between phytate and P_A indicating that improvement of P bioavailability in maize grain is possible with conventional plant breeding techniques aimed at both traits. Furthermore, the lack of correlations between P_A or phytate and other traits

important to maize production, particularly yield, is favorable for the improvement of grain P profiles and overall agronomic performance simultaneously.

A series of selection indices were constructed to investigate the potential of including phytate and P_A in ongoing recurrent selection programs. Selection differentials, being the difference between nine selected families (10% selection intensity) and the population mean, are presented in Table 4. Two of the indices constructed (heritability 1 and rank summation 1) first truncate the set to include the top 30 families based on yield, moisture, root lodging, and stalk lodging performance and assign new index values based on phytate and P_A evaluations. Although the methods used in this study were designed for high-throughput, extensive labor is still required for grinding and 96-well plate preparation and therefore a truncation method that minimizes the number of families being evaluated post-harvest would be desirable. As expected, these truncation-index methods apply thorough pressure to phytate and P_A in the desired directions, but put less emphasis on the other traits. Nevertheless, selection differentials are in the desired directions for yield, moisture, and root and stalk lodging indicating that a truncation and index combination is a reasonable selection method. Furthermore, truncation on traits evaluated in the field would increase the number of families that can be evaluated with a reasonable amount of labor required after harvest, which would most likely increase selection pressure. Table 4 shows that selection on an index value involving only phytate and P_A or either trait alone results in unfavorable changes in the agronomic traits essential to the successful development of a cultivar despite the lack of correlations between the P traits and agronomic traits (Table 4). The tremendous amount of work required to develop a cultivar with a favorably altered P profile would be in vain without the consideration of these agronomic traits in the breeding process. Also, because of

unfavorable correlations between protein and phytate and protein and yield, protein is expected to decrease with the use of any selection method investigated. Kernel weight is expected to increase with the use of each selection method.

In summary, this study was designed to report the H^2 of phytate and available P measured with rapid protocols for use in maize breeding programs. The findings indicate that both traits are heritable and could be improved simultaneously in conjunction with yield, root lodging, stalk lodging, and moisture. However, the phytate H^2 was the lowest among the traits measured, which was most likely due to large amounts of error resulting from the assay used rather than a lack of genetic variation. Refinement of laboratory techniques for increased precision will be continually pursued to improve the heritability of phytate and thus selection response. Due to the tissue pattern deposition of phytate in the seed, positive phytate:protein and negative phytate:starch correlations were found. Therefore, selection aimed at decreasing phytate may simply alter the germ:endosperm ratio of the grain.

Table 1. Constitution of standards used for phytate and available P (P_A) prediction. The tabled values underneath the subheadings "Conc." were calculated from the phytate and P_A stock solution concentrations and quantity of stock in each standard.

	Phytate			P_A		
Standard	HCl	Stock	Conc.	HCl	Stock	Conc.
	———μL———		μg mL^{-1}	———μL———		μg mL^{-1}
1	1000	0	0	1000	0	0
2	970	30	137	970	30	3
3	965	35	160	960	40	4
4	960	40	182	950	50	5
5	955	45	205	920	80	8
6	950	50	228	900	100	10

Table 2. Estimated variance components (family = σ_F^2, family by environment = σ_{FE}^2, error = σ_e^2), family by environment F-values, broad-sense heritabilities, and the mean, minimum, and maximum values for each trait.

		Statistic							
Trait	Unit	σ_F^2	σ_{FE}^2	σ_e^2	F value†	H^2	Mean	Min	Max
Phytate	μg mL^{-1}	35.40	7.19	83.30	1.22	0.59	181	158	201
P_A	μg mL^{-1}	0.77	0.04	0.44	1.22	0.86	4.24	2.44	8.08
Yield	Mg ha^{-1}	0.81	0.17	0.62	1.54**	0.77	6.69	3.49	9.25
Moist	g kg^{-1}	366.0	49.0	86.9	2.14**	0.89	200	160	265
Root Lodging	%	110.0	36.4	37.1	2.72**	0.80	9.21	0	56.90
Stalk Lodging	%	4.71	1.29	4.68	1.52*	0.72	2.51	0	15.90
Test Weight	kg m^{-3}	750.0	14.0	297.0	1.10	0.90	757	669	837
Protein	g kg^{-1}	32.70	2.02	9.59	1.52*	0.91	90.2	78.3	108.0
Oil	g kg^{-1}	3.18	0.26	1.29	1.35	0.87	39.1	35.3	45.6
Starch	g kg^{-1}	36.80	3.34	13.00	1.56**	0.88	591	576	606
Kernel Weight‡	g 250 K^{-1}	31.7	0.71	6.05	1.24	0.94	66.0	51.5	79.2

† F-values calculated for family × environment interaction source of variation.

*,** Significant at the 0.05 and 0.01 probability levels, respectively. Significance indicated for family by environment interaction F-value only.

‡ Kernel weight units are grams per 250 whole kernels.

Table 3. Phenotypic (above diagonal) and genotypic (below diagonal) correlations among traits based on S1 family means from the BS31 population. Units for each trait are presented in Table 2.

Trait	Phytate	P_A	Yield	Moisture	Root lodging	Stalk lodging	Test weight	Protein	Oil	Starch	Kernel weight	Silk emergence
Phytate		0.05	-0.14	-0.04	-0.17	0.16	0.16	0.37**	-0.07	-0.33**	-0.17	0.09
P_A	0.15		-0.15	-0.03	0.13	-0.17	0.18	0.09	0.01	-0.03	0.13	0.15
Yield	-0.12	-0.10		0.02	-0.12	0.15	-0.05	-0.46**	0.08	0.36**	0.27*	-0.35**
Moisture	-0.10	-0.10	0.19		0.24*	-0.35**	-0.71**	-0.03	-0.34**	0.22*	0.33**	0.42**
Root lodging	-0.16	0.11	-0.06	0.26		-0.17	-0.26*	0.07	-0.14	-0.02	0.04	0.12
Stalk lodging	0.27	-0.20	0.26	-0.47	-0.16		0.25*	-0.01	0.06	-0.06	-0.16	-0.08
Test weight	0.30	0.19	-0.11	-0.73	-0.33	0.30		0.15	0.31**	-0.25*	-0.34**	-0.38**
Protein	0.51	0.04	-0.53	0.00	0.16	0.05	0.16		-0.02	-0.88**	-0.02	0.09
Oil	-0.10	0.00	0.07	-0.42	-0.19	0.04	0.39	-0.03		-0.40**	-0.20	-0.03
Starch	-0.45	0.02	0.44	0.23	-0.07	-0.05	-0.30	-0.88	-0.40		0.15	-0.05
Kernel weight	-0.25	0.13	0.32	0.35	0.11	-0.10	-0.41	-0.05	-0.29	0.20		-0.01
Silk emergence†												

*,** Significant at the 0.05 and 0.01 probability levels, respectively.

† Silk emergence units are the number of days after planting to midsilk emergence. Genotypic correlations for silk emergence were not estimated because measurements were taken in only one environment.

Table 4. Tabled values are selection differentials (10 percent selection intensity) for each trait when the indices described in the methods section are used. High values for P_A, yield, test weight, protein oil, starch, and kernel weight were considered favorable. Low values for phytate, moisture, root lodging, stalk lodging, and silk emergence were considered favorable.

	Trait											
Index	Phytate	P_A	Yield	Moisture	Root lodging	Stalk lodging	Test weight	Protein	Oil	Starch	Kernel weight	Silk emergence
	— μg mL^{-1}—		Mg ha^{-1}	g kg^{-1}	%	%	kg m^{-3}	——— g kg^{-1} ———			g 250 K^{-1}	DAP†
Heritability 1	-4.63	1.01	0.41	-14.90	-4.92	-0.88	19.30	-2.15	0.03	2.28	4.39	-1.77
Heritability 2	-2.04	0.83	0.92	-18.30	-7.55	-0.70	17.90	-2.88	1.33	0.61	3.89	-1.81
Rank summation 1	-8.43	0.61	0.39	-7.69	-4.76	-0.85	5.14	-3.52	0.49	2.97	5.77	-1.09
Rank summation 2	-7.72	0.35	1.05	-9.28	-6.46	-1.39	9.94	-2.79	1.11	0.98	4.98	-2.14
Method 5	-3.32	0.99	0.81	-13.50	-5.71	-0.67	15.60	-3.32	0.63	2.06	5.36	-1.87
Method 6	-2.84	0.98	0.85	-18.10	-2.48	0.31	16.30	-1.58	0.86	-0.13	2.78	-2.02
Method 7	-7.05	1.11	0.60	4.50	2.36	-1.64	-6.44	-2.90	0.08	2.94	5.12	-0.54
Heritability on phytate and P_A	-7.80	1.40	-0.21	5.25	10.98	-1.04	-5.63	-1.02	-0.50	2.17	3.24	0.90
Phytate	-13.70	0.01	0.01	5.53	8.18	-1.54	-20.10	-4.30	-0.09	4.37	2.91	-0.20
P_A	0.23	1.79	-0.10	-3.97	3.38	-0.71	16.40	0.43	-0.35	1.05	2.86	0.68
Max‡	-13.70	1.79	1.71	-30.20	-9.02	-2.51	50.20	11.10	3.70	11.00	10.00	-3.60

† DAP – days after planting to midsilk emergence.

‡ Selection differential for each trait when selections are made on that trait alone.

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CHAPTER 5: EFFECT OF LAB REPLICATION AND DESIGN ON THE GENETIC DETERMINATION OF PHYTATE AND AVAILABLE PHOSPHORUS IN A MAIZE BREEDING POPULATION

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ABSTRACT

It would be desirable to decrease phytate and increase available P concentrations in maize grain to help alleviate associated nutritional and environmental problems simultaneously. However, the study of any metric character requires the reliable measurement of many individuals. Precision can be increased through experimental design and replication, but the latter increases experiment size and thus resources. Alpha row-column designs, a form of incomplete blocking, are commonly used in large variety trials to account for experimental unit heterogeneity. Grain quality traits, such as phytate and available P, are frequently measured on 96-well plates that resemble fields in their rectangular shapes and experimental unit matrix. It was the objective of this study to examine the effects of lab replication and row-column analysis on lab repeatability, field level heritability, and family selections. Available P measurements were highly repeatable (0.93 to 0.97) and repeatability was only slightly increased by including row and column effects in the model. On the other hand, phytate was less repeatable (0.46 to 0.75) and repeatabilities were substantially increased by including row and column (0.71 to 0.81). Available P field level heritability was only slightly increased by the inclusion of additional lab replication and the inclusion of row and column effects in the analysis. Phytate

heritability was improved considerably from the model including the least factors to the most (0.38 to 0.59). When lab replications were analyzed as row-column designs as opposed to RCB designs, sets of selected families and selection differentials were slightly different. We suggest that breeders and experimenters wishing to measure quality traits in the laboratory consider experimental design and analysis to account for as many sources of variation as possible to maximize quality of data from less repeatable assays and ultimately make the best selections.

INTRODUCTION

Cereal grains and oil seeds have particularly high concentrations of phytate (O'Dell, 1972), which is known to have negative impacts on the environment in addition to human and animal nutrition. Because monogastric animals such as swine and poultry lack the enzyme phytase, most of the P in their maize based diets passes into the feces and onto the landscape where it contributes to surface water eutrophication (Sharpley et al., 1994). For example, the bioavailability of P in maize is less than 15% (Cromwell and Coffey, 1991). Also, phytate is a strong chelator of important minerals such as iron and zinc (Erdman, 1981) and therefore contributes to human micronutrient deficiencies in developing countries (Raboy, 2002).

Many of the problems associated with P in maize grain are not due to the concentration of total P per se, but rather to the fact that most of the P is bound in phytate (Raboy, 2001). Therefore, it would be desirable to increase the amount of available P (P_A) and reduce the amount of phytate in maize grain. The study of any metric character requires the measurement of many individuals. Thus, if the measurement of a trait involves elaborate

and laborious procedures, the study of any such trait becomes impractical (Falconer and Mackay, 1996). Furthermore, the success of breeding is contingent upon the ability to differentiate germplasm and lines in a rapid, inexpensive manner that can be incorporated into normal breeding operations. Methods have been developed for the measurement of phytate in plant material, grain samples, and feed stuffs, but most are laborious and expensive requiring heating, cooling, multiple round of centrifuging, or specialized equipment (Vaintraub and Lapteva, 1988). An available P assay derived from Chen et al., (1956) has been used to qualitatively identify low-phytic acid mutant lines (Raboy et al., 2000), but quantitative determinations of P_A in maize have not been reported to the knowledge of the authors. Because repeatability sets an upper limit to heritability, precision of phytate and P_A measurements should compare well with other traits commonly quantified and modified in maize. Additional replication can increase the precision of experiments (Cochran and Cox, 1957), but also add to their size thus requiring more resources. The use of alpha lattices, a form of incomplete blocking, is another approach to increasing precision through accounting for experimental unit heterogeneity and requires little additional input thanks to modern statistical software and computing power (Yau, 1997). Alpha lattices, hereafter referred to as row-column designs, are commonly used in large field experiments to reduce environmental variation, but are commonly overlooked when measuring quality traits in the laboratory. Grain quality data are frequently obtained from 96-well plates which resemble fields in their fixed rectangular shape and experimental unit matrix (wells). It is desirable to know if randomizing and analyzing 96-well plates like large variety trials could add precision to experiments and improve field level heritability. One objective of this study was to estimate the repeatability of phytate and P_A measurements taken with rapid and simple

protocols on 96-well plates analyzed as a row-column design and randomized complete block (RCB) design. By obtaining field plot values for phytate and P_A through different types of laboratory data analysis, it was possible to investigate the impact of such factors on phytate and P_A field level genetic determination, selection differentials, and probability of correct selection. The results of this study could be of relevance to any breeder or experimenter wishing to account for extra sources of variation during the measurement of grain quality traits.

MATERIALS AND METHODS

Plants from the BS31 population were randomly self-pollinated to produce 90 S_1 families. These 90 families were evaluated in a randomized complete block design with two replications each at two Iowa locations (Ames and Carroll) during the summer of 2004. Plots consisted of two rows 5.49 m long with 0.76 m between rows. The plots were overplanted by machine and thinned to a uniform plant density of 62 140 plants ha^{-1}. The lines were allowed to open pollinate and an approximate 454 g grain sample was obtained from each field plot by an automated sampler during machine harvest. A subsample of 30 whole kernels was taken from each field plot sample and milled so the 70% of the millings passed through an 80 micron mesh screen. Phytate and P_A were measured by the modified assays of Vaintraub and Lapteva (1988) and Raboy et al. (2000) respectively as previously described by Lorenz (2005). Measurements were made on 96-well plates randomized in row-column designs (Cycdesign, 2002) with three lab replications within each field block; each lab replication contained six standards along with all 90 plots in a field block. The replications consisting of 96-well plates within field blocks will be referred to as "lab blocks" and

experimental units at the laboratory level will be referred to as "wells". Field experimental units will be referred to as "field plots".

Statistical Analysis

The data set from this experiment consists of two levels, lab and field, and adjustments for outliers were made at each level. Prediction of phytate and P_A concentrations in each well through regression on standard concentrations was performed as previously described by Lorenz (2005). Lab repeatability was estimated for each field block and indicates the reliability of measurements taken within a single field plot subsample. Lab repeatability (r_L) was calculated with $r_L = \sigma_F^2 / [\sigma_F^2 + (\sigma_e^2 / r)]$, where σ_F^2 is the family variance, σ_e^2 is the error variance, and r is the number of lab blocks ($r = 3$).

Field plot phytate and P_A values were estimated five different ways (lab analysis):

1) Taken from a single lab block.
2) Taken as the least-square mean (LSmean) across any two lab blocks.
3) Taken as the LSmean across any two lab blocks with row and column nested within lab block fit as random factors (analyzed as row-column design).
4) Taken as the LSmean across all three lab blocks.
5) Taken as the LSmean across all three lab blocks analyzed as a row-column design.

Field plot values from each lab analysis were then used for field level broad-sense heritability estimates on a family mean basis:

$$H^2 = \frac{\sigma_F^2}{\sigma_F^2 + (\sigma_{FE}^2 / e) + (\sigma_e^2 / re)}$$

where σ_F^2 is the family variance at the field level, σ_{FE}^2 is the variance due to family by environment interaction, σ_e^2 is the error variance at the field level, e is the number of environments ($e = 2$), and r is the number of field blocks per environment ($r = 2$). All variance components were obtained through the restricted maximum likelihood procedures of PROC MIXED (SAS Institute, 2003a). The Wald Z-test was used to test if the variance components were significantly different than zero (SAS Institute, 2003b). Selections (10% selection intensity) were made on family means across environments for each lab analysis. The field plot values assigned with lab analysis 5 were assumed to be closest to the true values. Selected families from the other lab analyses were identified and selection differentials were calculated from the family means based on the data set of lab analysis 5. Finally, the selections made through lab analysis 5 were assumed to be correct and therefore the probability of correct selection by the other methods was calculated.

RESULTS AND DISCUSSION

Measurements on P_A within field plots were highly reliable with r_L values ranging from 0.93 to 0.97 across the four field replications. Lab repeatability was only increased slightly when row and column were included in the analysis. One the other hand, phytate r_L values were substantially lower ranging from 0.46 to 0.75. When row and column were fit, r_L values increased substantially (0.71 to 0.81) mainly due to large decreases in the residual variance. Lattice designs are typically more effective for experiments with larger amounts of residual variance (Yau, 1997). The partitioning of variance to rows and columns may be a result of several artifacts in the lab: reagents and supernatants are transferred and added

column by column through a multi-channel pipette; tissue samples are placed in wells column by column; the spectrophotometer, which analyzes whole plates, may spatially bias readings. Lab repeatabilites for each field block are displayed in Table 1.

Experimental design and number of replications in the laboratory directly affect heritability in the field. To determine the importance of these factors in phytate and P_A selection, field level broad-sense heritability estimates (H^2) were made for each lab analysis outlined in the *Materials and Methods* section of this paper. Also, selection differentials and probabilities of correct selection for each lab analysis were calculated. Family and family by environment effects were highly significant ($P < 0.01$) and nonsignificant ($P > 0.05$) respectively for all lab analyses and both traits. With regard to phytate, H^2 was increased by 55% when three lab blocks constituted a field plot value compared to one lab block (lab analysis 1 to 4 and 5). The row-column analysis over both two and three lab blocks decreased error, but an increase in σ^2_{FE} compromised an improvement in field level H^2. Although σ^2_{FE} estimates were not found to be significantly different than zero, no explanation can be made for the increase in this variance by the row-column analyses of lab blocks. Because P_A was determined to be highly repeatable within field plots, H^2 was only increased by 6% from lab analysis 1 to lab analysis 5. Nevertheless, fitting the row-column model did slightly increase field level H^2 (0.84 to 0.86) with regard to three lab blocks, but the significance of a such a small increase is certainly dubious. Variance components (σ^2_F, σ^2_{FE}, and σ^2_e) and H^2 estimates for each analysis and trait are displayed in Table 2. Another way of looking at the effects of selecting on one, two, or three lab blocks is to observe the selection differentials achieved for each scenario. The best information was assumed to be

from the lab analysis which fit three lab blocks and row-column. Family selections were made based on the other lab analyses, but the means of the selected groups and selection differentials were calculated from the "best information" data sets. Regarding phytate, it is apparent that increasing lab blocks and the row-column analysis improves selection differentials. Also noteworthy is the discrepancy between selected entries and selected means of lab analyses 4 and 5 despite their equal H^2s. Available P showed only slight improvements in selection differentials upon using more lab blocks and different analyses. However, the probabilities of correct selection (probability of selecting entry also selected by "best information" model) indicated that slightly different sets of selected families are obtained using different analyses. Therefore, number of lab blocks and design may have a final impact on selection regardless of measurement repeatability.

Spatial designs and numerous replications are commonly used in large variety trials to control environmental variation and obtain the most accurate estimates of variety performances, but are not commonly used for the measurement of quality traits in the laboratory. The objective of the present study was to determine the effects of lab replication and design on field level H^2 estimates and selection outcomes by simply calculating different field plot values through different lab analyses and reporting simple statistics such as field level H^2s, selection differentials, and "correct" selection probabilities. The results of the present study regarding the row-column randomization and analysis of 96-well plates to obtain phytate and P_A measurements suggest that such an approach may increase precision in the lab and ultimately at the field level. The results of less repeatable measurements (phytate) were more dramatic. Highly repeatable measurements (P_A) showed only a slight improvement in H^2 and selection differentials with an increase in lab replication and the

fitting of row-column effects. Thus, the increase in P_A field level precision may not justify the extra labor of increasing lab replication. Finally, because randomization and analysis to account for spatial effects (row and column in this case) by modern statistical software hardly increases labor and expense, it is suggested that such an approach be considered for less precise grain quality assays in order to maximize data quality.

Table 1. Lab repeatabilities for phytate and available P when 96-well plates were analyzed as randomized complete blocks (RCB) and row-column designs (row-col) within each field replication. Lab repeatability measures the precision of measurements taken from within single field plot subsamples.

Trait	Field block 1	Field block 2	Field block 3	Field block 4
Phytate (RCB)	0.72	0.75	0.61	0.46
Phytate (row-col)	0.81	0.80	0.71	0.72
Available P (RCB)	0.95	0.94	0.93	0.97
Available P (row-col)	0.96	0.96	0.97	0.98

Table 2. Estimated field level variance components (family = σ_F^2, family by environment = σ_{FE}^2, error = σ_e^2) and broad-sense heritabilities for phytate and available P. Analyses were performed on field plot values consisting of least-squares means obtained from the analyses (model) described in the *Materials and Methods*.

	Phytate			
Model	σ_F^2	σ_{FE}^2	σ_e^2	H^2
1. One lab block†	30.03	0	192.34	0.38
2. Two lab blocks (RCB)†	33.51	0	120.72	0.53
3. Two lab blocks (row-col)†	37.69	13.86	117.58	0.51
4. Three lab blocks (RCB)	34.50	0	96.25	0.59
5. Three lab blocks (row-col)	39.09	10.78	87.92	0.59
	Available P			
Model	σ_F^2	σ_{FE}^2	σ_e^2	H^2
1. One lab block†	0.74	0.05	0.58	0.81
2. Two lab blocks (RCB) †	0.73	0.05	0.48	0.83
3. Two lab blocks (row-col)†	0.76	0.04	0.48	0.84
4. Three lab blocks (RCB)	0.73	0.06	0.44	0.84
5. Three lab blocks (row-col)	0.77	0.04	0.44	0.86

† Variance components and H^2 are averages of three estimates.

Table 3. Means of selected families and selection differentials (10% selection intensity) when selections were made on family means derived from plot values consisting of least-squared means obtained from the different lab analyses. Entry means used to calculate the selection means and differentials in each scenario were taken from the model that included three lab blocks analyzed in a row-column design. Lower and higher values for phytate and available P, respectively, were considered favorable.

	One lab block	Two lab blocks (RCB)	Two lab blocks (row-col)	Three lab blocks (RCB)	Three lab blocks (row-col)
Phytate					
Selected mean	171.15	169.49	168.06	168.08	166.94
Selection diff.	-10.26	-11.92	-13.37	-13.35	-14.48
Prob.†	0.48	0.59	0.70	0.78	1.00
Available P					
Selected mean	6.00	6.05	6.05	6.04	6.07
Selection diff.	1.76	1.81	1.81	1.80	1.84
Prob.†	0.74	0.81	0.89	0.78	1.00

† Probability of "correct" selection. Correct selections were assumed to be made by the lab analysis that included three lab blocks analyzed as row-column lattices.

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CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

The results found and presented in the preceding chapters suggest that available P and phytate can be evaluated as metric characters through simple and rapid techniques. The broad-sense heritability estimated for available P (0.86) in chapter 4 indicates that the qualitative assay of Raboy et al. (2000) can be modified and used to quantitatively determine available P with a relatively high degree of precision. Although, significant differences were found between inbred lines and S1 families with regard to phytate, the high estimates of residual variance stress the need for further refinement of the protocol to increase precision and thus phytate heritability. Some possible solutions include allocating resources to decrease available P lab replications and increase phytate replication, dilution and/or purification of grain extracts, and extraction of larger amounts of grain (some unpublished data revealed that 15 mg extractions may separate lines better than 10 mg extractions). Those who continue research into phytate evaluation for breeding purposes should become very familiar with the phytate analytical literature before extensive amounts of time are spent in the laboratory. It is also suggested that fine tuning of phytate evaluations be conducted on a limited number (perhaps 10) of inbred lines preferably grown in the same field experiment. F-values corresponding to the line source of variation should be maximized for the chosen assay derivation.

The family by environment interaction source of variation was determined to be nonsignificant. However, this favorable result should be investigated further through testing across years and more diverse locations. Another notable finding included an estimated phytate:available P correlation coefficient near zero. While many studies have reported high

phytate:total P correlations, a correlation between phytate and available P has not been published to the knowledge of the author and has important implications for breeding programs striving to improve both traits simultaneously. Significant correlations between phyate and protein (r = 0.37 to 0.51) and phytate and starch (r = -0.33 to -0.45) were also found and are in accordance with previous reports and literature discussing the distribution of phytate in the seed. These findings together with the phytate:kernel weight relationship discussed in chapter 3 persuade the author to hypothesize that long-term selection for decreased whole kernel phytate concentration would result in kernels with a larger endosperm to germ ratio. Raboy et al. (1989) also briefly mentioned the relationship between seed organ sizes and phytate levels. The lack of unfavorable correlations between the P related traits and important agronomic traits in the BS31 population contributed to the finding that progress (estimated by selection differentials) could be made for phytate, available P, yield, moisture, and root and stalk lodging with the use of various selection indices and index-truncation combinations. Protein and kernel weight are expected to decrease and increase, respectively. Selection on phytate and available P alone would assuredly result in unfavorable changes with respect to essential agronomic traits and therefore a multiple trait selection method is advocated to avoid the painful realization of useless germplasm. Because the rank-summation index/truncation combination made adequate progress for all considered traits and more families may be evaluated in the field with little or no increase in post-harvest labor, this approach should be seriously considered in ongoing selection for improved phytate and available P levels. Because only a fraction of selection differentials are expected to be maintained from generation to generation (Falconer and Mackay, 1996), it is immediately obvious that long-term commitment and perseverance

is needed for noticeable progress. Such a situation is even more apparent when phytate and available P levels of the *lpa* mutant lines are envisioned as end goals. On a positive note, any favorable improvement in phytate and available P would be valuable (Chad Stahl, 2005, personal communication).

Finally, increased lab replication may not be efficient for highly repeatable assays such as available P, but field level precision of the phytate assay was shown to benefit greatly. Analyzing 96-well plates as row-column lattices improved lab repeatability with respect to phytate. Field level heritability was not affected by the use of row-column lattice analyses on the 96-well plates. However, phytate field level error variance was decreased by the inclusion of row and column, but an unexplainable increase in family by environment variation compromised any improvement in heritability. Furthermore, sets of selected families at the field level were different across different lab analyses indicating that experimental design and analysis at the laboratory level can impact final family selections. Because powerful randomizations and analyses using modern software and computers hardly increase time and labor, it is suggested that such approaches be considered when striving to optimize simple, but less than perfect grain quality assays.

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