This is to certify that the

thesis entitled

PHOSPHORUS, PHYTIC ACID AND ZINC CONTENT IN SEEDS OF DRY BEANS (PHASEOLUS VULGARIS, L.): THEIR INTER-RELATIONSHIPS, INHERITANCE AND RANGE IN VARIABILITY AMONG GENOTYPES

presented by

Karen Ann Cichy

has been accepted towards fulfillment
of the requirements for

M.S. degree in Plant Breeding and Genetics

George L. Hosfield
Major professor

Date May 28, 2002

PHOSPHORUS, PHYTIC ACID, AND ZINC CONTENT IN SEEDS OF DRY BEANS (*PHASEOLUS VULGARIS* L.): THEIR INTERRELATIONSHIPS, INHERITANCE, AND RANGE IN VARIABILITY AMONG GENOTYPES

By

Karen Ann Cichy

A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE

Plant Breeding and Genetics Program

2002

ABSTRACT

PHOSPHORUS, PHYTIC ACID, AND ZINC CONTENT IN SEEDS OF DRY BEANS (*PHASEOLUS VULGARIS* L.): THEIR INTERRELATIONSHIPS, INHERITANCE, AND RANGE IN VARIABILITY AMONG GENOTYPES

By

Karen Ann Cichy

Phytic acid, the major form of phosphorus in bean seeds, is considered an antinutrient in humans because it reduces the bioavailability of zinc. Plant breeding for increased seed zinc and reduced phytic acid is one strategy to improve the zinc nutritional value of the bean. Accordingly, experiments were conducted with two navy bean cultivars that differed in seed zinc content. 'Voyager' (high seed Zn) and 'Albion' (low seed Zn) were grown under different combinations of P and Zn fertilizer. An inheritance study was also conducted with the same cultivars. Parents were intermated to produce F_1, F_2, and backcross generations. The seed of the fertilizer and inheritance studies was harvested and screened for phytic acid, P, and Zn levels. The results of the fertilizer study indicated that seed Zn levels were higher in Voyager than Albion at 2 and 4 mg g^{-1} soil Zn in combination with all P fertilizer treatments. The results of the inheritance study indicated variability for seed zinc levels. The trait also showed high heritability. An additional 48 bean genotypes were also screened for phytic acid and Zn levels. There was limited variability in seed phytic acid levels, but substantial variability in seed Zn levels among the genotypes.

ACKNOWLEDGEMENTS

I am grateful to the members of my guidance committee: Dr. James Kelly, Dr. Bernard Knezek, and Dr. Maurice Bennink, for their advice, suggestions, interest, and criticism.

I would also like to thank my major advisor, Dr. George Hosfield, for his support and guidance. He gave me the opportunity to conduct the research in an area of plant genetics of particular interest to me.

I am very grateful to Ken Grafton and John Moraghan at North Dakota State University. They provided me plant material and very valuable assistance in understanding the nature of the trait and guidance in reviewing the literature.

I would like to thank also the many people in the USDA, including Shawna Bushey, Robyn Engleright, Missy Stiefel, and Dr. Robert Olien, who provided technical assistance in many aspects of my work and who also made coming to work each day enjoyable.

I would like to thank my parents and my brothers and sister for their support and understanding. I am also thankful to my husband to be, David Mota-Sanchez for his faith in me. I would also like to thank the many friends I made while at Michigan State University who created a wonderful environment in which to work and live.

TABLE OF CONTENTS

LIST OF TABLES.....vi

LIST OF FIGURES.....viii

INTRODUCTION AND LITERATURE REVIEW.....1

Introduction.....1

Literature Review.....2

Phytic acid, its Function in Seeds, Structure, Biosynthesis, Breakdown, and Physiological role.....2

Role of Phytic Acid in Human Nutrition.....10

Phytic Acid and Zinc Bioavailability.....10

Phytic Acid and Protein Interaction.....14

Phytic Acid and the Environment.....14

Ways to Reduce Phytic Acid in Foods.....15

Seed Preparation.....16

Plant Growing Conditions and Genetic Intervention.....17

CHAPTER 1: PHYTIC ACID, PHOSPHORUS, AND ZINC ACCUMULATION AND THEIR INTERACTIONS IN THE SEED OF A ZINC EFFICIENT AND NON EFFICIENT NAVY BEAN CULTIVAR.....21

Introduction.....21

Materials and Methods.....24

Results and Discussion.....27

Conclusions.....40

CHAPTER 2: INHERITANCE OF SEED PHYTIC ACID, PHOSPHORUS AND ZINC LEVELS IN THE SEED OF NAVY BEAN.....43

Introduction.....43

Materials and Methods.....45

Results and Discussion.....51

Seed Phytic Acid Phosphorus Concentration.....51

Seed Non Phytic Acid Phosphorus Concentration.....57

Seed Zinc Concentration.....59

Conclusions.....60

CHAPTER 3: SCREENING OF PHASEOLUS VULGARIS L. GENOTYPES FOR VARIABILITY IN PHYTIC ACID AND ZINC IN RAW AND THERMALLY PROCESSED SEED.....62

Introduction.....62

Materials and Methods.....63

Results and Discussion.....66

Conclusions.....73

APPENDIX.....74

LITERATURE CITED.....84

LIST OF TABLES

Table 1. Mean phytic acid phosphorus concentration [PA-P], non-phytic acid phosphorus concentration [non PA-P], and total phosphorus concentration [P] in Voyager and Albion navy bean cultivars grown under three levels of P and five levels of Zn fertilizer.....28

Table 2. Means for phytic acid phosphorus (PA-P) content per experimental unit (seed per 3 plants), non-phytic acid phosphorus (non PA-P) content per experimental unit and total phosphorus (P) content per experimental unit in Voyager and Albion navy bean cultivars grown under three levels of P and five levels of Zn fertilizer 30

Table 3. Means for seed yield, seed zinc concentration [Zn] and zinc content per experimental unit (Zn) (seed of three plants) in Voyager and Albion navy bean cultivars grown under three levels of P and five levels of Zn fertilizer.....36

Table 4. The percent of phosphorus (P) in the phytic acid (PA) form in seed of Voyager and Albion navy bean cultivars grown under three levels of phosphorus and five levels of zinc fertilizer.....38

Table 5. The regression relationship (R^2) between three phosphorus (P) treatments added to the soil and the concentrations of phosphorus in phytic acid (PA-P) and non-phytic acid (non PA-P) forms in seed of two navy bean cultivars.....39

Table 6. Multiple linear regression for the effect of cultivar and P and Zn added to the soil on phytic acid phosphorus (Pa-P), non-phytic acid phosphorus (non Pa-P), and zinc (Zn) in the seed of two navy bean cultivars.....39

Table 7. Coefficients of gene effects in generation means analysis.....50

Table 8. Mean levels of total seed phosphorus (P), the phytic acid (PA-P) and non-phytic acid fractions of phosphorus (non PA-P), the percent of total seed phosphorus in the phytic acid form (%PA-P) and seed zinc (Zn) in two navy bean cultivars and four additional generations established by an initial cross between the cultivars.....52

Table 9. Chi-square test for the adequacy of an additive/dominance generation means model for the concentration of seed phytic acid phosphorus [PA-P], non-phytic acid phosphorus [non PA-P], and zinc [Zn] in a cross between Voyager and Albion navy bean cultivars and four additional generations established by an initial cross between the cultivars 55

Table 10. Estimates and standard error of genetic effects in the generation means analysis model for the concentration of seed phosphorus [P], phytic acid phosphorus [PA-P], non phytic acid phosphorus [non PA-P], percent phytic acid phosphorus [% PA-P] and zinc [Zn] in a cross between Voyager and Albion navy bean cultivars and four additional generations established by an initial cross between the cultivars.....56

Table 11. The variance components and heritability estimates of phytic acid phosphorus [PA-P], non-phytic acid phosphorus [non PA-P], total phosphorus [P] and zinc [Zn] concentration in the seed derived from a population developed from a cross between Albion and Voyager navy bean. 56

Table 12. The commercial class, seed coat color, seed weight and yield of 48 dry bean genotypes screened for the concentration of phytic acid phosphorus, total phosphorus, and zinc in the seed.....67

Table 13. The concentration of zinc [Zn], phosphorus [P], and the phytic acid form of phosphorus [PA-P] in raw and thermally processed seed of 48 dry bean genotypes.....69

LIST OF FIGURES

Figure 1. The chemical structure of phytic acid as a chair conformation.....2

Figure 2. Phytic acid biosynthesis pathway.....6

Figure 3. Phytase action on phytate in seeds.....8

Figure 4. Comparison of the concentrations of phosphorus in phytic acid [PA-P] and non- phytic acid [non PA-P] forms in seed of Voyager (Voy) and Albion (Alb) navy bean cultivars grown under three levels of P (0, 60, and 120mg P kg^{-1}) and five levels of Zn (0, 0.5, 1, 2, and 4 mg Zn kg^{-1}) fertilizer. Fig. 4A shows the responses for the two cultivars for seed phosphorus in the phytic acid [PA-P] form. Fig. 4B shows the responses for the two cultivars for seed P in the non phytic acid [non PA-P] form.....29

Figure 5. Comparison of the content per experimental unit (seed of 3 plants) of phosphorus in phytic acid (PA-P) and non-phytic acid (non PA-P) forms in seed of Voyager (Voy) and Albion (Alb) navy bean cultivars grown under three levels of P (0, 60, and 120mg P kg^{-1}) and five levels of Zn (0, 0.5, 1, 2, and 4 mg Zn kg^{-1}) fertilizer. Fig. 5A shows the responses for the two cultivars for seed phosphorus in the phytic acid (PA-P). Fig. 5B shows the responses for the two cultivars for seed non PA-P.....31

Figure 6. Seed yield per experimental unit (3 plants) of Voyager (Voy) and Albion (Alb) navy bean cultivars grown under three levels of P (0, 60, and 120mg P kg^{-1}) and five levels of Zn (0, 0.5, 1, 2, and 4 mg Zn kg^{-1}) fertilizer.....33

Figure 7. Correlations between the concentration of zinc [Zn] and the concentration of phosphorus in the phytic acid form [PA-P] in the seed of Albion and Voyager navy beans grown under three levels of P (0, 60, and 120mg P kg^{-1}) and five levels of Zn (0, 0.5, 1, 2, and 4 mg Zn kg^{-1}) fertilizer.....42

Figure 8. Frequency distribution of phosphorus concentration [P] in Albion and Voyager navy bean seed and four generations derived from the intermating of Albion and Voyager. The arrows indicate the mean [P] of each generation.....53

Figure 9. Frequency distribution of phytic acid phosphorus concentration [PA-P] in Albion and Voyager navy bean seed and four generations derived from the intermating of Albion and Voyager. The arrows indicate the mean [PA-P] of each generation.....54

Figure 10. Frequency distribution of the non-phytic acid phosphorus concentration [non PA-P] in Albion and Voyager navy bean seed and four generations derived from the intermating of Albion and Voyager. The arrows indicate the mean [non PA-P] of each generation.....58

Figure 11. Frequency distribution of zinc concentration [Zn] in Albion and Voyager navy bean seed and four generations derived from the intermating of Albion and Voyager. The arrows indicate the mean [Zn] of each generation.....61

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Dry bean (*Phaseolus vulgaris* L.) is an important food source worldwide, and is a staple for many people in Latin America and Africa and those on vegetarian diets in developed countries. Beans are nutritionally valuable because they are high in complex carbohydrates (~ 40%) and protein (19 to 33 %), low in fat, high in dietary fiber and are a good source of iron, calcium, thiamine, folic acid, and niacin (Shellie and Hosfield, 1991). Despite dry bean's nutritional benefits, the crop is underutilized because of several antinutrients that limit food quality (Jaffe, 1973). Phytate, the main phosphorus storage form in legume seeds, is one such antinutrient (Barampama and Simard, 1993; Oberlas, 1975). Phytate tightly binds divalent cations and reduces their availability and utilization in humans and animals. Zinc is the cation most tightly bound to phytate. Studies have shown that zinc bioavailability in vivo is considerably less than the actual zinc content of the seed (World Health Organization, 1996).

Reduction of seed phytate in dry bean may be possible through genetic manipulation or by altering the growing conditions or processing methods of seeds. Accordingly, a study was conducted to: (1) Determine the effects of phosphorus and zinc fertilizer treatments on total seed phosphorus, phytic acid, and zinc in a zinc efficient and a zinc inefficient navy bean cultivar. (2) Ascertain the inheritance of seed phosphorus, phytate, and zinc in a zinc efficient and inefficient cultivar using generation means analysis. (3) Screen a select sample of dry bean cultivars and breeding lines to identify the range in variability in phytic acid, phosphorus, and zinc

content in raw and cooked bean seed for plant breeding purposes. (4) Propose a plant breeding strategy to increase seed zinc while simultaneously reducing phytate levels in bean seed.

LITERATURE REVIEW

Phytic Acid, its Function in Seeds, Structure, Biosynthesis, Breakdown, and Physiological Role:

Phytate (*myo*-inositol 1,2,3,4,5,6 hexa*kis*phosphate) is a storage form of phosphorus found in all plant seeds; it is most abundant in cereal and legume seeds (Oberlas, 1983). Sixty to eighty percent of the total phosphorus in legume seeds is stored as phytate. Phytate has a strong negative charge that is maintained over a wide pH range. The molecule has 6 phosphate groups (Fig.1) that can covalently bind to cations, especially K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+} (Tsao, 1997). These cations, along with phosphorus and *myo*-inositol form a pool used by seedlings during the early stages of development.

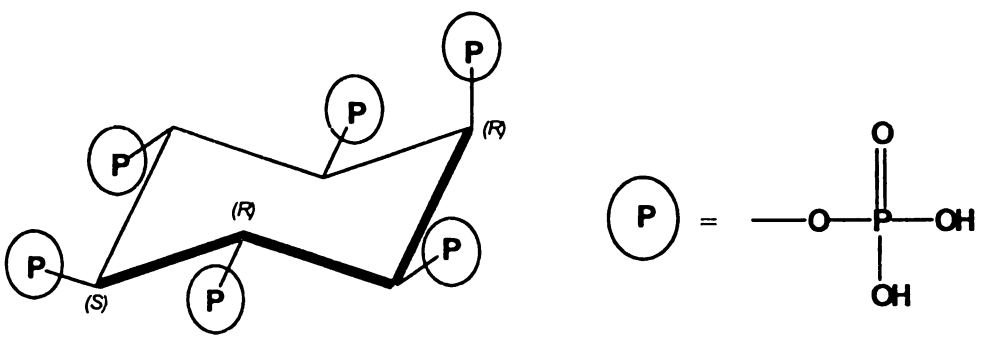

Figure 1. The chemical structure of phytic acid (chair conformation)

The types of cations stored as part of a phytate complex differ among species and differ within diverse cells of the same species (Lott et al., 1993). A study conducted by Reddy and Pierson (1987) found that in great northern bean (*P. vulgaris*) calcium, magnesium, and potassium were most frequently stored with phytate (6.4mg/g, 20.5mg/g and 2.6mg/g respectively). Phytate complexes containing calcium are water insoluble. In great northern bean, 40% of the total phytate was water-insoluble and the other 60% was in a water-soluble form (Reddy et al., 1988). Iron, zinc, and sodium are also stored as part of the complex, but in much lower concentrations (528μg/g, 133μg, and 157μg g^{-1} respectively) as expressed on a dry weight basis (Reddy et al., 1988).

In the seed of common bean, the majority of phytate is located in the cotyledons, typically within protein bodies, which are membrane bound vacuoles 1.5 to 8 μm in diameter (Lott et al., 1993). In great northern bean, protein bodies contained 34.3% protein, 30% carbohydrates, and 26.6% phytic acid (Reddy and Pierson, 1987). Inside the protein bodies, phytate makes up electron dense particles called globoid crystals (Lott and Buttrose, 1977). Globoid accumulation occurs during the maturation phase of seed development, which is the phase of rapid seed reserve synthesis and accumulation (Lott et al., 1993). In rice (*Oryza sativa* L.), globoid accumulation has been shown to occur as early as four days after anthesis, which is early for a seed reserve storage molecule. This fact suggests phytic acid may serve a role during seed development, in addition to its role in providing nutrients to the growing seedling (Yoshida et al., 1999).

Biosynthesis of phytic acid is believed to take place in the same tissue where it is stored. Organ et al. (1988) demonstrated that phytate synthesis in castor bean (*Ricinus communis*) occurred in embryonic tissue in the presence of exogenous carbon and phosphorus. Under such conditions, phytate synthesis was not a temporary phenomenon, but occurred throughout seed development in response to added phosphate, even during post germination growth. Greenwood and Bewley (1984) suggested that in castor bean synthesis occurs in the cytoplasm outside the developing protein bodies on cisternal endoplasmic reticulum, and following synthesis, phytate is transported to protein bodies via endoplasmic reticulum vesicles. The conclusions drawn by these authors were based on light microscopy and energy dispersive x-ray analysis data. A study in rice (O. sativa) also indicated phytate biosynthesis occurs in the embryo soon after anthesis. The enzyme that catalyzes the production of myo-inositol 1 phosphate from glucose-6-phosphate, myo-inositol 1 phosphate synthase, appears to direct phytate biosynthesis (Yoshida et al., 1999).

There is a growing literature regarding how phytate biosynthesis occurs. The pathway was first elucidated in the cellular slime mold, *Dictyostelium* (Stephens and Irvine, 1990). Phytic acid biosynthesis occurs via stepwise phosphorylation of *myo*-inositol (3) P. (abbrev. Ins (3) P). Stephens and Irvine (1990) found the biosynthesis to proceed as follows: Ins (3) P → Ins (3,6) P_2 → Ins (3,4,6) P_3 → Ins (1,3,4,6) P_4 → Ins (1,3,4,5,6) P_5 → InsP_6. By 1996, the pathway was elucidated in the plant kingdom. In the organism *Spirodela polyrhiza*, Brearly and Hanke (1996a,b) found the pathway for phytate biosynthesis to be: Ins (3) P → Ins (3,4) P2 → Ins (3,4,6) P_3

→ Ins (3,4,5,6) P_4 → Ins (1,3,4,5,6) P_5→ $InsP_6$, which was different than the pathway in *Dictyostelium*. Interestingly, lower inositol phosphates, $InsP_1$ through $InsP_4$ apparently have a role as intracellular messengers in plants and animals (Urbano et al., 2000), but in the phytic acid biosynthesis pathways elucidated in *Dictyostelium* and *S. polyrhiza*, there was no obvious direct link for the intermediates to function as signal molecules (Brearley and Hanke, 1996b). This indicates that the lower inositol phosphates involved in signaling may be phosphorylated at different positions of the inositol ring than are the intermediates of phytic acid.

The branch of the pathway prior to the stepwise phosphorylation of Ins (3) P is the cyclization of D-glucose-6-phosphate to Ins (3) P, catalyzed by D-*myo*-inositol (3) P1synthase (MIPS) (Figure 2.). This step is not unique to phytic acid biosynthesis (Loewus, 1990). In fact, most of the synthesized Ins (3) P is dephosphorylated to free *myo*-inositol. *Myo*-inositol is an important sugar in plants and is a precursor to compounds with a diverse array of functions including signal transduction, stress response, cell wall structure, and hormonal homeostasis (Loewus and Murthy, 2000). Despite its role in numerous cellular processes, characterization and cloning of the MIPS enzyme gene has been helpful in understanding the role of phytic acid in the plant. The MIPS gene was cloned in a number of plant species, including *Arabidopsis thaliana* (Johnson 1994), *Citrus paradisii* (Abu-Abied and Holland, 1994), *Mesembryanthemum crystallinum* (Ishitani et al., 1996), *Nicotiana tabacum* (Hara et al., 2000), *P. vulgaris* (Wang and Johnson, 1995), and *Glycine max* (Hegeman et al., 2001). The availability of the sequence has made possible experiments that demonstrate the role of the MIPS enzyme in myo-inositol synthesis,

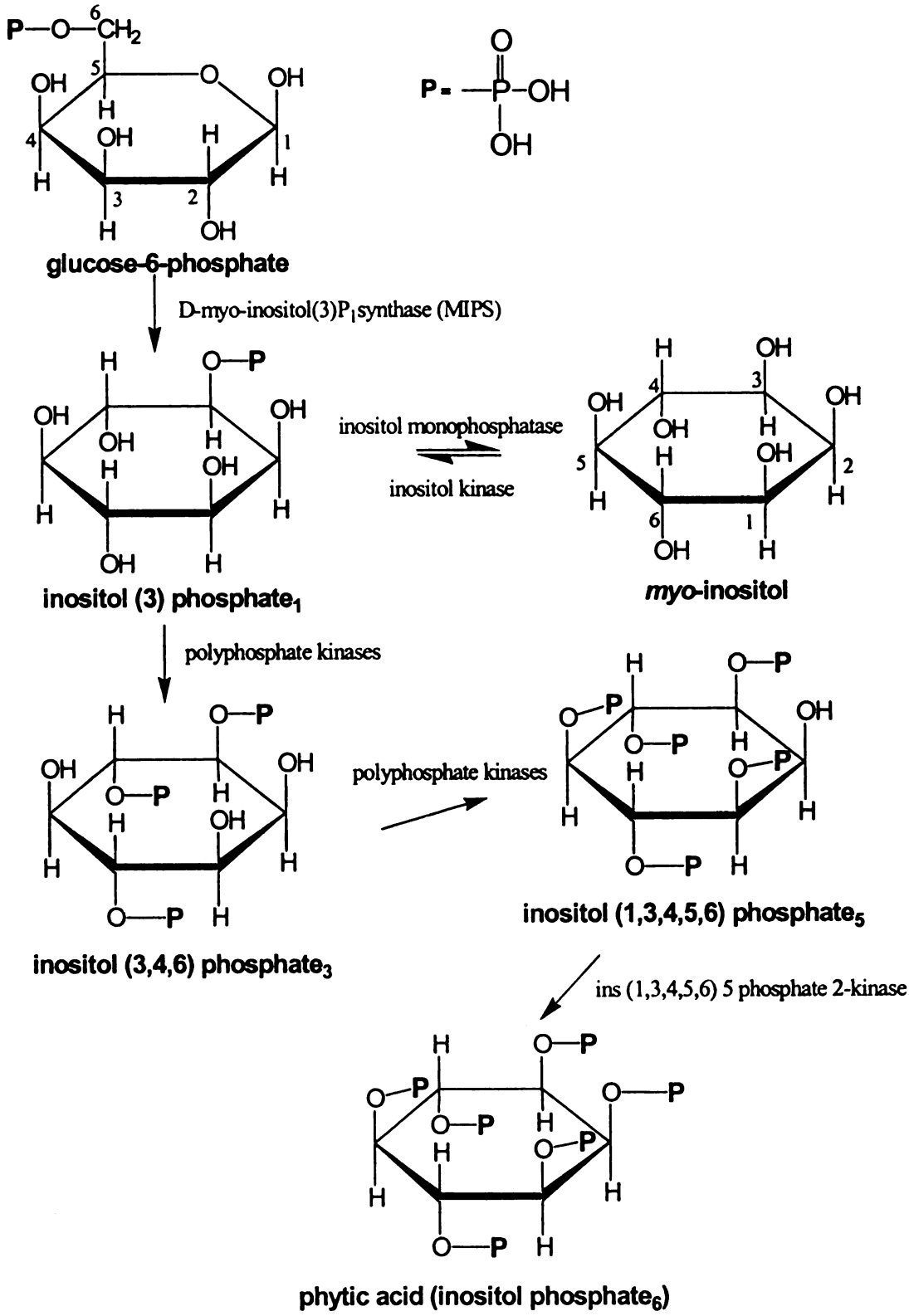

Figure 2. Phytic acid biosynthesis pathway

and the central role of myo-inositol in the plant. Keller et al. (1998) used antisense technology to develop transgenic potato (*Solanum tuberosum* L.) lines with the MIPS gene knocked out. These transgenic lines had a 93% reduction in leaf inositol levels compared to the wild type. Raffinose and galactinol levels were reduced by 90%. Additional altered phenotypes were also observed in the transgenic plants, including altered leaf morphology, reduced apical dominance, and early leaf senescence (Keller et al., 1998).

Phytate breakdown occurs in germinating seeds. Phytate is hydrolyzed into inorganic P, lower P esters of myo-inositol, free myo-inositol, and cations by a class of nonspecific phosphomonoesterases called phytases (Reddy et al., 1989). Phytases catalyze the stepwise removal of the inorganic orthophosphate from phytate. Data obtained from monocot seed phytases of spelt (*Triticum spelta* L.), rye (*Secale cereale* L.), barley (*Hordeum vulgare* L.), and oat (*Avena sativa* L.) presents a generalized scheme for the action of phytases (Fig. 3) (Greiner and Alminger, 2000). The lower inositol phosphates produced by the action of plant, microbial, and fungal phytases are different from inositol phosphate intermediates of the phytic acid biosynthetic pathway (Brearley and Hanke, 1996b). The 3-phytases appear to be characteristic of microorganisms, they act by hydrolyzing $InsP_6$ to Ins (1,2,4,5,6) P5, with the exception of *Paramecium* and *Escherichia coli*, which both have 6-phytases (Greiner and Alminger, 2000). In the seeds of higher plants, 4-phytases or 6-phytases act on phytic acid (Cosgrove, 1980). Most plant phytases, including 4-phytases and 6-phytases, have optimal activity at low pH.

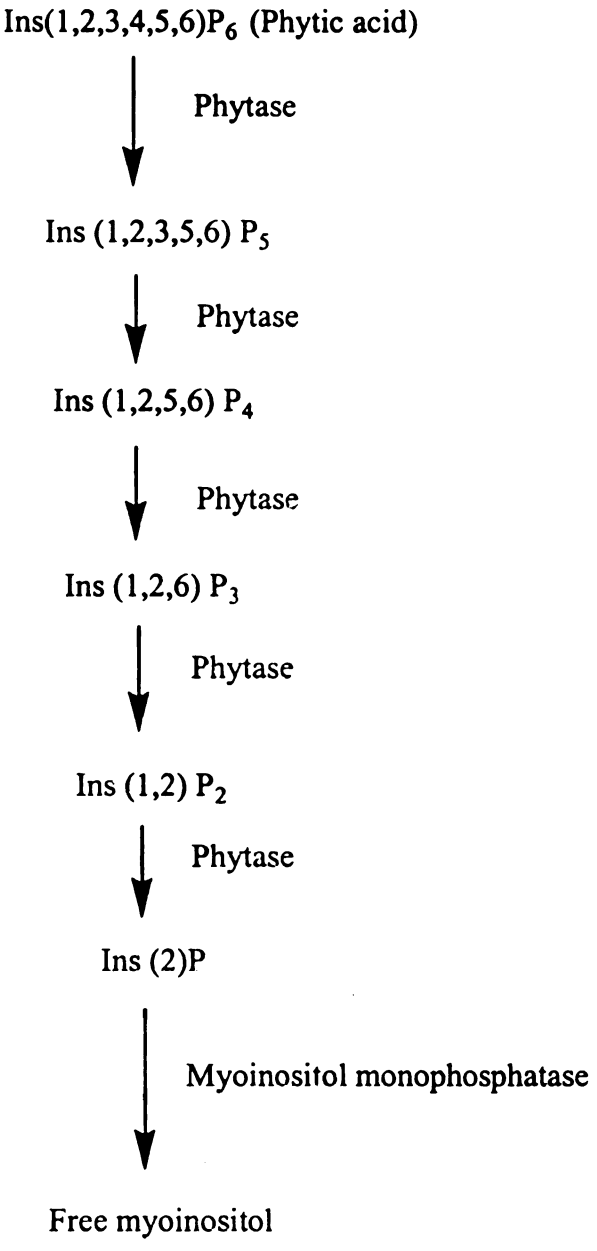

Figure 3. Phytase action on phytate in seeds (Greiner and Alminger, 2001)

Barrientos et al. (1994) characterized a 5-phytase in lily (*Lilium longiflorum* L.) pollen with a pH 8 optimum. Unlike acid phytates, this alkaline phytase could not dephosphorylate beyond $InsP_3$. The $InsP_4$, $InsP_3$, and $InsP_2$ isomers produced by the alkaline phytase also differ from those of the acid phytates, and to this date, the physiological significance of these intermediates has not been established.

There is a small amount of enzymatic activity in non-germinated seeds, but phytases are most active during germination. Lolas and Markakis (1977), showed that the phytase activity of navy bean (*P. vulgaris*) increased slightly during the first two days of germination, followed by a 7-fold increase in activity from day 2 to day 6 of germination. A gradual decrease in activity after day 6 was noted (Lolas and Markakis, 1977). The optimal temperature for phytase activity in navy bean was 50°C. Seeds may contain pre-existing phytase, but additional phytase is synthesized during germination (Cosgrove, 1980). Phytase induction and synthesis appeared to be activated, in part, by low orthophosphate levels in germinating seedlings (Gibson and Ullah, 1990) Similarly, synthesis and activity of phytase was inhibited by high levels of orthophosphate (Kikunaga et al., 1991).

In addition to its importance as a cation storage molecule, phytic acid has recently been shown in potato (*S. tuberosum*) to be a component of a Ca^{2+} dependent signaling pathway of stomata guard cells that inhibits K^+ inward flux (Lemtiri-Chlieh at al., 2000). Additional undiscovered functions of phytic acid may exist in plants.

Role of Phytic Acid in Human Nutrition

Much of the interest in phytate has traditionally been based on its role in human and animal nutrition. Under human physiological conditions, some phytate complexes are insoluble, whereas others are soluble during digestion. The liberated phytic acid that was part of the soluble complexes forms new complexes with minerals that are present in the gut (Schlemmer et al., 1995). This process decreases the bioavailabity of phosphorus and cations, especially iron and zinc, stored as a part of the complex.

Phytic acid and Zinc Bioavailability:

Numerous studies, using a variety of experimental techniques, have shown a correlation between high phytate diets and limited zinc absorbance in the gastrointestinal tract of humans and animals (Saha et al., 1994; House et al, 1982; Turnlund et al., 1984; Sandstrom et al, 1988; Lonnerdal et al., 1988; Hunt et al., 1998; Zhou et al., 1992). Zinc is a micronutrient required in the human diet. The recommended daily allowance established for people of the United States is 12 to 15 mg for adults and 10 mg for children. However, this amount of zinc may be too low for people who consume phytate rich vegetarian diets. Zinc deficiency in humans is widespread, and is due not only to the zinc content of the diet, but also to its bioavailability. Although extreme zinc deficiency is rare, marginal deficiency has been documented in North and South America, and parts of Europe, Asia, and Africa (Welch, 1993). Based on national food balance data collected by the FAO, it is estimated that 49% of the human population is at risk for inadequate zinc in their diet (International Zinc Association, 2000). Marginal zinc deficiency in humans can

cause dermatitis, a weakened immune system, prolonged wound healing, taste dysfunction, impaired dark vision, diarrhea, anorexia, decreased learning capacity, and depression. Zinc deficiency can also cause abnormal fetal development, premature births, and reduced growth rates of infants and children (Hambidge, 2000).

Dialysis is one method used to estimate Zn bioavailability by simulating human digestion *in vitro*. In addition to *in vitro* studies, *in vivo* studies have been conducted to determine the effect of phytate on zinc bioavailability in laboratory animals, typically rats. In vivo experiments involved feeding intrinsically labeled ^{65}Zn to rats as part of controlled diets, followed by measuring the radioactivity to determine Zn absorption. Similar zinc radioisotope experiments can be safely conducted in humans. Radionuclide labeled ^{65}Zn can be used with whole body counting to measure post-prandial Zn absorption (Wise, 1995). Stable isotope ^{67}Zn can be safely used in humans. In addition, in animals and humans, nutritional balance studies have been conducted to estimate absorption by feeding a know amount of zinc in the diet and measuring the amount in the feces (Wisker et al., 1991). Numerous zinc absorbance studies in humans and animals have indicated that the phytic acid/zinc molar ratio (moles phytate/moles zinc) is useful to predict possible zinc deficiency based on composition of the diet (Torre, 1991) (Oberlas and Harland, 1981). Typically, total diet phytate/zinc molar ratios of greater than 12 to 15 indicate chemical zinc deficiency (Reddy et al., 1989).

The majority of experimental evidence for the inhibitory effect of phytic acid on zinc absorbance has been conduced in laboratory animals. House et al, (1982)

combined ^{65}Zn tracing and nutritional balance studies in rats to examine how the addition of 2% sodium phytate to a diet affected zinc bioavailability. These authors (House et al., 1982) observed a decrease in true zinc absorption from 46.8% in the basal diet to 32% in the phytate diet. Zhou et al., (1992) found that reducing the amount of endogenous phytate in soybean by limiting phosphorus to the growing plant or by altering processing methods increased zinc bioavailability in weanling rats. The results were based on the measurement of the tibia zinc content of the rats (Zhou et al., 1992). Saha et al. (1994) studied Zn bioavailability in rats with intrinsically labeled whole-wheat flour. These authors (Saha et al., 1994) also measured endogenous phytate instead of sodium phytate. Diets that were 0.19% phytate had higher zinc absorption than diets that contained 1.64% phytate, whereas there was no difference in zinc absorbance between diets that contained 1.64% and 1.85% phytic acid.

Since phytic acid ($InsP_6$) is just one of the inositol phosphates found in grain and legumes seed, Lonnerdal et al (1989) looked specifically at how phytic acid and each of the lower inositol phosphates affected ^{65}Zn absorption in rats. Phytic acid ($InsP_6$) was found to strongly inhibit zinc absorption; $InsP_5$ exhibited decreased inhibition compared to $InsP_6$, whereas $InsP_3$ and $InsP_4$ showed no inhibition, indicating reduced dephosphorylation of phytic acid can positively affect zinc absorption.

Although laboratory animals are used to understand the relationship between phytate and zinc bioavailability in humans, animal diets are different from those of humans and extrapolations should be made with caution (Wise, 1995). Studies on human subjects have been conducted to a lesser extent than for animals. Turnlund et

al. (1984) used stable isotope ^{67}Zn to determine zinc absorbance in four adult human males fed controlled diets for 63 days that varied over time in sodium phytate and α-cellulose levels. Average apparent zinc absorbance was 34% in the basal diet with a range of 21.9% to 51.3%. With the addition of 2.34g of phytate to the diet, an amount equivalent to a phytate/zinc molar ratio of 15, the apparent mean zinc absorbance fell to 17.5% with a range of 14.2% to 24.9%. Sandstrom et al. (1989) used ^{65}Zn in humans to study the effect of various animal and plant protein sources on zinc absorbance. Sandstrom et al. (1989) found that after the addition of fish or chicken as a protein source to a bean (*P.vulgaris*) meal, the zinc absorbance increased 50 to 70% while the total zinc content remained constant. Hunt et al. (1997) compared zinc absorbance with extrinsically labeled ^{65}Zn from human subjects who maintained lacto-ovo vegetarian and omnivorous diets over an eight-week period. The subjects who maintained the non-vegetarian diet had molar ratios of phytate to zinc of less than five while subjects who maintained the lacto-ovo vegetarian diet had phytate to zinc molar ratios of 5 to 15. The zinc content of subjects maintaining the typical lacto-ovo vegetarian diet was estimated to be 10-30% less than subjects maintaining a non-vegetarian diet and subjects maintaining the lacto-ovo vegetarian diet absorbed 35% less zinc. The results of these studies (Sandstrom et al., 1989, and Hunt et al., 1997) indicate zinc deficiency would likely be greater in people who maintain vegetarian diets with a phytate to zinc molar ratio greater than 15 than non-vegetarians. Molar ratios greater than 15 are often found in diets with limited animal protein and protein from grain and legumes (Hunt et al., 1997).

Phytic Acid and Protein Interaction:

Although phytic acid is best known for its role in reducing micronutrient bioavailability, phytic acid has been implicated (Cheryan, 1980) in reducing protein utilization as well. Two factors are involved in reduced protein utilization due to phytic acid: First, phytic acid can form complexes with proteins, which may decrease their solubility. Interactions between phytic acid and protein are most prevalent at low pH (Cheryan, 1980). Second, phytic acid may reduce activity of protein digestive enzymes. *In vitro* studies showed that phytic acid reduced the activity of the proteases pepsin and trypsin (Knuckles et al., 1989), (Vaintraub and Bulmaga, 1991). However, *in vivo* studies showed no effect of phytic acid on protein digestive enzymes; the involvement of phytic acid in protein bioavailability is inconclusive. Vaintraub and Bulmaga (1991) speculated that the discrepancy between the in vitro and in vivo studies may have been due to differences in the formation of phytate, enzyme, protein, or substrate complexes in each of these systems, and most likely were affected by pH changes.

Phytic Acid and the Environment:

One side effect of the limited digestibility of phytate by monogastric animals is that the majority of phosphorus in feed made from cereal or legume seeds is excreted. For example, 80% of the P in a typical corn diet fed to swine and poultry is in the form of phytate. The P is unavailable to these animals because they lack the phytases necessary to hydrolyze phytate (Ertl et al., 1998). Animal waste is the principle source of phosphorus pollution in agricultural systems (Ertl et al., 1998). The average P in animal manures was estimated as follows: beef -5.6 g kg^{-1}; dairy-

11.7 g kg^{-1}; poultry layers-20.8 g kg^{-1}; broilers-16.9 g kg^{-1}; sheep-10.3 g kg^{-1}; swine-17.6 g kg^{-1}; and turkeys-16.5 g kg^{-1}. Phosphorus pollutes watersheds via runoff and erosion of agricultural lands and causes eutrophication of streams, rivers, and lakes. This form of pollution reduces value and use of surface water for fisheries, recreation, industry and drinking (Sharpley et al., 1994). Lott et al. (2000) estimate that 4.1 billion metric tons of field crops, fruits, and seeds are produced annually, which contain 35 million metric tons of phytic acid - 9.9 million tons of which are phosphorus. Lott et al. (2000) used these estimates in conjunction with the FAO fertilizer yearbook (1995), and calculated that about 65% of the P fertilizer sold in the world ends up as phytic acid phosphorus. This figure represents a significant loss of P from soils, crops, and animals. Ertl et al. (1998) studied the effects of feeding swine and poultry diets consisting of low phytic acid corn. These authors (Ertl et al., 1998) found that corn with normal levels of phytic acid contained 3.8 g kg^{-1} total P with 84% in the form of phytic acid, while the low phytic acid corn contained 3.9 g kg^{-1} total P and only 33% as phytic acid. The animals fed the low phytic acid corn diet had a 9 to 40% reduction in fecal P. Low phytate crop varieties may hold the answer to effectively reducing the P pollution in the environment. This type of work is in the preliminary stages and in the instance of Ertl et al's work (1998), the low phytic acid cultivars also had a significant yield reduction.

Ways to Reduce Phytic Acid in Foods:

One way to ameliorate the problems caused by phytate in human and animal nutrition and the environment it to reduce its levels in food. Scientists have

examined genetic variability, plant production conditions and post harvest seed handling as possible means to reduce seed phytic acid.

Seed Preparation:

Preparation, cooking, and processing methods all influence the amount of phytate present in foods. Schlemmer et al. (1995) found that soaking soya bean (*Soja hispida* Max.), mung bean (*Phaseolus aureus* Roxb.), brown bean and white bean (*P. vulgaris*), green pea and yellow pea (*Pisum sativum* L.), or lentils (*Lens esculenta* Moench) for 8, 16, or 24 hours at 22°C had no affect on phytic acid levels in the seed. A number of other studies (Reddy et al., 1989; Greiner and Konietzny, 1998) have also shown that soaking does not change seed phytic acid levels in legumes. The effect of standard cooking in boiling water for two to three hours has been more variable. Schlemmer et al. (1995) found that cooking caused the hydrolysis of a minimal amount of the phytic acid to $InsP_5$. On the other hand, Greiner and Konietzny (1998) found that cooking reduced the phytic acid content in beans 16 to 24% with a corresponding increase in $InsP_5$, $InsP_4$, and $InsP_3$. Thermal processing (canning) of foodstuffs has also been examined as a means to reduce phytic acid, but results were conflicting in regard to effect. For example, Schlemmer et al. (1995) found canning had no effect, whereas Tabekhia and Luh (1980) showed a 70 to 75% reduction in phytic acid with canning in beans (P. *vulgaris*).

The processing methods that have lead to the sharpest decline in phytic acid are those that activate phytase. For example, a 10-hour, 60°C water bath heat treatment (optimal temperature of phytase) of navy bean caused a 90% drop in seed phytic acid (Chang et al., 1977). Fermentation also activates endogenous phytases of

yeast and other microorganisms that significantly reduce phytic acid. Kannan et al. (2001) found fermented black bean (*P. vulgaris*) had 10% less phytate than the non-fermented control. Conversely, a number of compounds sometimes used in the fermentation process, especially calcium and magnesium salts have been shown to inhibit phytate hydrolysis, thereby preventing a significant reduction in seed phytic acid (Reddy et al., 1989). One way to circumvent the inactivation of phytase during fermentation is to add exogenous phytase to food. Phytase is a common additive of animal feed used to improve the diet and limit phosphorus excretion in manure (Ertl et al., 1998).

Plant Growing Conditions and Genetic Intervention:

The principal environmental factor that affects the content of seed phytic acid is available soil P. A strong linear relationship between P fertilizer levels and seed phytic acid concentration has been shown in the seed of a number of crops including, soybean (*G. max*) (Lolas et al., 1976) wheat (Asada et al., 1969) and oats (*O. sativa*) (Miller et al., 1980). The reduction of P fertilizer is not a practical way to reduce phytic acid levels because phosphorus is required for plant growth and seed yield (Raboy, 1985).

Genetic factors that influence P uptake and translocation also influence seed phytic acid content (Raboy and Dickinson, 1993). Screening of dry bean (*P. vulgaris*) genotypes from North America, Central America, South America, Europe, and Africa has revealed significant variability in seed phytic acid levels (19.6 to 27.5 $\mu mol\ g^{-1}$) (Welch et al., 2000). In order to reduce seed phytic acid without also reducing seed total P, seed phytic acid specific genetic factors must be identified.

When one only examines the variability in phytic acid among cultivars of a species, it is impossible to determine if the differences are due to factors influencing P uptake or phytic acid synthesis (Raboy and Dickinson, 1993).

It has been possible to separate genetic factors controlling total seed P from phytic acid P. Mutation breeding has been used to develop low phytic acid lines in maize (*Zea. mays*) (Larson and Raboy, 1999), barley (*Hordeum vulgare* L.) (Larson et al., 1998), rice (*O. sativum*) (Larson et al., 2000), and soybean (*G. max*) (Wilcox et al., 2000). Raboy et al. (1990) studied a series of mutations in maize that affected germ and aleurone tissue. These authors found that certain germ mutations had low levels of phytic acid and abnormally high concentrations of inorganic phosphorus, suggesting tissue specificity for phytic acid synthesis in maize. Two non-allelic low phytic acid mutants were found in maize and barley, and they were classified as lpa1 and lpa2 (Larson et al., 1998; Raboy et al., 2000). The lpa1 mutant showed a 66% reduction in phytic acid phosphorus and a corresponding increase in inorganic P. The lpa2 mutants similarly had a 50% reduction in phytic acid P and a corresponding increase in Pi, but unlike the lpa1 mutants, the lpa2 mutants also showed an increase in lower inositol phosphates compared to the wild type. Larson and Raboy (1999) hypothesize that the lpa1 mutant interrupts an early step in the phytic acid biosynthesis pathway, possibly the MIPS gene or a MIPS regulatory gene. The lpa2 mutant, on the other hand, appeared to affect a step later in the pathway. A genetic mapping experiment showed that one lpa1 mutant of maize mapped to same region of chromosome 1S of maize; thus providing additional evidence that the mutation affected the MIPS gene. In barley, the lpa1 mutant did not map to the same region of

the genome as MIPS, suggesting this mutant may be a trans-acting factor (Larson and Raboy, 1999). Raboy et al. (2001) tested low phytic acid maize mutants to determine their agronomic performance. Fourteen near-isogenic maize hybrid pairs were created with an lpa1 mutant or its non-mutated counterpart serving as a parent in each of the pairs. The mutants had no effect on germination, stalk strength, flowering date, or grain moisture at harvest, but each of the mutant classes showed a 4% to 23% reduction in seed yield as compared to the wild type. An animal feeding study with the grain showed that the low phytic acid mutants had 50% more available P that resulted in a 50% reduction in P waste (Spencer et al., 2000).

The best strategy to reduce seed phytate levels may be by finding and using seed specific promoters to the MIPS or other genes in the phytic acid pathway. There is evidence in soybean for the existence of a family of MIPS genes important in different plant parts. Hageman et al. (2001) found four DNA sequences that hybridized to the MIPS clone. One of the sequences, GmMIPS1, was expressed in immature cotyledons and was developmentally regulated indicated by its reduced expression levels as the seed matured.

Low phytic acid mutants have been identified in many major grain and legume crops, including maize, rice, barley, and soybean. These crops are staples in the diet of humans and animals around the world. Dry bean is a valuable, protein rich legume missing from this list. The identification of a low phytic acid genotype in this crop would be very useful for the development of bean cultivars with improved micronutrient bioavailability. The detection and selection of beans with increased density of zinc is also an important strategy to improve the micronutrient value of the

bean seed. Preliminary studies are necessary to understand the interaction of phytic acid and zinc in beans under different environmental conditions, the inheritance of zinc and phytic acid levels in the bean seed, and the variability of these seed components in existing germplasm. Once these essential steps are conducted, the best suited procedure to develop a bean with increased zinc density and decreased phytic acid density should be straightforward to identify.

CHAPTER 1: PHYTIC ACID, PHOSPHORUS, AND ZINC ACCUMULATION AND THEIR INTERACTIONS IN THE SEED OF A ZINC EFFICIENT AND NON-EFFICIENT NAVY BEAN CULTIVAR

INTRODUCTION

Improving the zinc nutritional value of dry bean (*Phaseolus vulgaris* L.) for human consumption requires an understanding of the relationship among phosphorus (P), phytic acid (PA) and zinc (Zn) in the seed. Phytic acid is a storage form of phosphorus found in all plant seeds; it is most abundant in cereal and legume seeds (Oberlas, 1983). PA is a polyanion at physiological pH and an effective chelator of nutritionally important mineral cations such as calcium, zinc, and iron (Raboy et al., 2001). The binding of Zn by PA in beans can contribute to zinc deficiency in human populations (Hambidge, 2000).

The relationship between P and Zn has been studied in the soil, and in roots and shoots of numerous plant species, including dry bean. However, neither the P and Zn relationship, nor the PA, P, and Zn relationship has been studied in dry bean seeds. Although, genetic differences for plant response to low zinc soils have been identified in bean, the responses to P and Zn fertilizers have not been characterized in the seed of this crop.

Uptake of Zn by plant roots occurs predominately through diffusion of the divalent cation (Zn^{+2}) from the soil solution. Soil type, pH, temperature and P level (Moraghan and Mascagni, 1991) influence zinc availability in the root zone.

Genotypic differences in susceptibility to deficient soil zinc of *P. vulgaris* were initially identified in studies with the navy bean cultivars Sanilac and Saginaw (Ambler and Brown, 1969). In low Zn soils, plants of both cultivars developed visual symptoms of Zn deficiency (necrotic and discolored leaves) although 'Sanilac' had the more severe symptoms. 'Sanilac' also had lower yield and reduced shoot Zn content compared to 'Saginaw', but at normal soil Zn levels, the two cultivars had comparable yields (Ambler and Brown, 1969). In addition, when grown in Zn deficient soils, 'Sanilac' had more P in the shoots than 'Saginaw', but 'Saginaw' had more P in the roots than 'Sanilac'.

Zinc efficiency is the term used to describe a genotype that has a higher yield than a standard ('check') genotype when grown in soils limiting in Zn for the check. 'Saginaw' is an example of a Zn efficient genotype and 'Sanilac' is an example of a Zn inefficient one. The Zn inefficiency trait in navy bean is believed to have originated in 'Sanilac'. This cultivar originated as an X-ray mutant and was the first navy bean cultivar with a determinant growth habit (Judy et al., 1965). 'Sanilac' was then used as a parent to transfer the determinacy trait into many other navy bean cultivars. Many of the early determinant navy bean cultivars are in the pedigrees of current day navy bean cultivars. 'Sanilac' was zinc inefficient and supposedly this trait was transferred to other navy beans along with 'Sanilac's' determinacy trait (Kelly, 2000).

Zinc efficient navy bean cultivars also accumulate higher levels of seed Zn than Zn inefficient ones. Moraghan and Grafton (1999) showed that 'Norstar' and 'Voyager'- both Zn efficient- and 'Albion' and 'Avanti'- both zinc inefficient- had

similar seed Zn concentrations in the absence of added Zn, but the efficient cultivars had 15 to 20% higher seed Zn than the inefficient cultivars when Zn was added to the soil. The response of 'Voyager' and 'Albion' seed [Zn] when Zn was added to the soil was observed under both greenhouse and field conditions. Under field conditions, with no added Zn, 'Albion' had the highest seed P of the four cultivars. This may indicate that the differences in Zn efficiency noted among the cultivars may be based on a plant's ability to limit excessive P uptake when Zn in the soil is limiting.

A high level of soil phosphorus is an environmental factor that can influence plant zinc deficiency in *P. vulgaris*, especially where available soil Zn is low. This phenomenon, known as P induced Zn deficiency is believed to be caused by a number of factors. The principle factor is a dilution effect such that the increased plant tissue growth due to the presence of added P causes a dilution of Zn concentration in the whole plant (Moraghan, 1980). Additional proposed mechanisms include: 1) P and Zn interact in the soil and limit zinc availability (Brown et al., 1970). 2) Phosphorus inhibits the translocation of Zn from roots to shoots (Singh et al., 1988). 3) Zinc deficiency promotes the increased transport of P from the roots to the leaves resulting in P toxicity symptoms (Loneragan et al., 1979 and Loneragan et al., 1982). 4) High P soils inhibit mycorrizal root infection, which decreases Zn uptake (Singh et al., 1986).

Detailed studies of P induced Zn deficiency in *P. vulgaris* have indicated possible mechanisms of the interaction. Singh et al. (1988) found the shoot concentration of Zn to decrease sharply as the amount of P added to the soil

increased, but the addition of P beyond 40 mg P kg^{-1} did not greatly influence Zn concentration further. McKenzie and Soper (1983) found that the presence of added Zn (8 mg Zn kg^{-1} soil) prevented a P induced reduction of Zn concentration in plant tissue. Zinc deficiency appeared to be caused by two factors. First, zinc concentration was diluted in plant tissue because added P fertilizer caused an increase in plant mass. Second, in the presence of high levels of P, there was restricted translocation of Zn from the roots to aboveground plant parts in low soil Zn in certain genotypes.

Since the Zn efficiency trait affects seed Zn concentration, it may also influence seed P and PA content, especially considering that increased P fertilizer causes a decrease in the concentration of Zn [Zn] in the seed. This study was undertaken because of the lack of information on how soil P and Zn affect [Zn] in the seed of *P. vulgaris*. In addition, Zn is an essential micronutrient for human nutrition whose bioavailability is reduced by phytic acid, and understanding the relationship between PA and Zn in the seed may be helpful for improving the Zn nutritional value of the seed. The objective of the study was to ascertain the relationship among seed P, PA, and Zn in a Zn efficient and Zn inefficient navy bean cultivars grown under different treatments of P and Zn fertilizers.

MATERIALS AND METHODS

'Voyager' and 'Albion' were the experimental material used in this study and are known to be Zn efficient and non-efficient cultivars, respectively. 'Voyager' and 'Albion' were grown in pots in a greenhouse at North Dakota State University. Five rates of Zn (0, 0.5, 1.0, 2.0, and 4.0 mg $ZnSO_4$-Zn kg^{-1}) and three rates of P (0,

60,120 Ca $(H_2PO_4)_2$-P kg^{-1}) fertilizer were applied in a factorial combination to each of the cultivars, for a total of 30 treatments. Each treatment was replicated four times, and the experiment was arranged in a randomized complete block design with pots as the experimental unit. The soil used in each pot was a Wheatville loam (coarse-silty over clayey, mixed over smectitic, superactive, frigid Aeric Calciaquolls) that had a pH of 8.2 and contained 12.2 g inorganic C kg^{-1}, 8.0 mg $NaHCO_3$-extractable P kg^{-1}, 0.35 mg DTPA-extractable Zn kg^{-1}, 4.5 mg DTPA-extractable Fe kg^{-1}, and 25 mg NO_3-N kg^{-1}. A basal dressing of 70 mg NH_4NO_3-N kg^{-1}, 90 mg K_2SO_4-K kg^{-1} was mixed with the soil before addition to the pots, and 1 mg $Na_2B_4O_7$-B kg^{-1} and 2 mg Fe EDDHA-Fe kg^{-1} were applied in liquid form to each pot. Eight seeds were planted per pot, and thinned to 3 plants per pot 7 days after emergence. Pots were watered as needed with distilled, deionized water.

Pods from each experimental unit (i.e., 3-plants per pot) were harvested when 80% of them had changed from green to pale yellow or brown (mature color). At the time of the final pod harvest, pod walls, stem tissue, and leaves were separated and oven dried at 50°C for 72 hours, weighed, ground in a stainless steel mill, and analyzed for Zn and P. Two subsamples of seed from the 3-plants of each pot were washed with distilled water, oven dried at 70°C for 24 hours, finely ground to fit through a sieve with 0.25 mm openings. Total P was measured by a molybdenum blue procedure and Zn was measured by atomic adsorption. For each analysis, 0.5 g of seed was ashed in a muffle furnace at 500°C for 6 hours. Next, 25ml of 3N HNO3 was added to the seeds. After one hour the mixture was filtered to remove undissolved particles. These samples were directly analyzed for Zn concentration by

atomic adsorption spectroscopy. For P analysis, the samples were diluted 10:1 v:v in 0.3N NaOH. Following the initial dilution, they were diluted again 15:1 v:v with 2.5N sulfuric acid containing 0.006% ammonium molybdate, 0.0002% antimony potassium tartrate, and 0.005% l-ascorbic acid. The sample absorbance was read on a Brinkmann dipping probe with a 880nm filter.

An additional subsample of seed was freeze dried, ground and analyzed for PA. Sample preparation for the analysis of phytic acid concentration [PA] in bean seed was conducted following the procedure of Lehrfeld (1980). Phytic acid was extracted from the samples by the addition of 3ml 0.5M HCl (trace element grade) to 0.100 g of sample. The acidified samples were mechanically agitated for 2 hours at 21°C. Samples were centrifuged at 12,000g for 15 minutes. The supernant was collected and diluted 1:5 (v/v) with distilled, deionized water. The diluted sample (15 ml total) was passed through a Bond Elut strong anion exchange column (Varian, Walnut Creek, CA) for purification. The column was washed once with 10 ml 0.05 M HCl, and PA was eluted with 3ml 2M HCl. The eluted fraction containing PA was air dried and dissolved in 5mM sodium acetate. The dissolved sample was filtered through a 2μm filter. Phytic acid levels in each sample were quantified by high performance liquid chromatography with a refractive index detector. The column used for analysis was a Waters Symmetry C18 column (3.9mm x 150mm) (Waters, Milford, MA) heated to a temperature of 40°C. Sodium acetate (5mM) was used as the solvent at a flow rate of 1.4 ml min^{-1}. Phytic acid dodecasodium salt from corn (*Z. mays* L.) (Sigma, St. Louis, MO) was used as a standard to determine

PA concentration. The PA values were divided by 3.55 to obtain P stored as PA (PA-P) values (Lott et al., 2000).

All statistical analyses were conducted using SAS software (SAS Institute, Cary, N.C., 1985). A paired t-test was used to determine differences between 'Albion' and 'Voyager' at different fertilizer treatments for seed P, PA-P, non PA-P, P as PA-P, Zn, and seed yield.

RESULTS AND DISCUSSION

Analyses of Variance, (Table A1) indicated that P and Zn fertilizer treatments and P x Zn interactions influenced the concentration of PA-P in the seed, but the cultivar effect for this character was not significant. The general trend noted for 'Albion' and 'Voyager' was that as P fertilizer increased, seed [PA-P] also increased and as Zn fertilizer increased, seed [PA-P] decreased except at the lowest level of P (Figure 4A & Table 1). The concentrations of seed P not stored in PA [non PA-P] and total seed P [P] were significantly affected by P and Zn fertilizer treatments and cultivar. The [P] and [non PA-P] were significantly affected by P x Zn, cultivar x Zn, cultivar x P, and cultivar x P x Zn interactions (Table A1). The [non PA-P] tended to increase as P fertilizer increased and decrease as Zn fertilizer increased (Table 1; Fig 4B). The striking difference between seed [non PA-P] and [PA-P] was that [PA-P] increased almost identically in 'Albion' and 'Voyager' as added P was increased but the [non PA-P] in the seed was cultivar dependent (Fig 4B). These results are clearly shown in Figure 4A and B at the 60 mg kg^{-1} soil and 120 mg kg^{-1} soil P fertilizer treatments.

Table 1. Mean phytic acid phosphorus concentration [PA-P], non-phytic acid phosphorus concentration [non PA-P], and total phosphorus concentration [P] in Voyager and Albion navy bean cultivars grown under three levels of P and five levels of Zn fertilizer.

Soil Treatment		PA -P		Sig. of a—b at	Non PA-P		Sig. of c—d at	Total P		Sig. of e—f at
P	Zn	Albion(a)	Voyager(b)	P ≤0.05†	Albion(c)	Voyager(d)	P ≤0.05†	Albion(e)	Voyager(f)	P ≤0.05†
mg kg^{-1}		g kg^{-1}			g kg^{-1}			g kg^{-1}		
0	0	1.3	1.4	NS	1.7	1.4	NS	3.0	2.8	NS
	0.5	1.4	1.3	NS	1.6	1.3	**	3.0	2.6	**
	1	1.4	1.3	NS	1.4	1.4	NS	2.8	2.7	NS
	2	1.4	1.2	NS	1.5	1.5	NS	2.9	2.7	NS
	4	1.4	1.5	NS	1.5	1.3	NS	2.9	2.8	NS
60	0	3.4	3.4	NS	3.3	2.3	**	6.7	5.7	**
	0.5	2.7	2.8	NS	2.5	1.8	**	5.2	4.6	**
	1	2.6	2.6	NS	2.3	1.7	**	4.9	4.3	**
	2	2.3	2.3	NS	2.2	1.7	**	4.5	4.0	**
	4	2.1	2.2	NS	2.2	1.8	**	4.3	4.0	**
120	0	3.5	3.6	NS	3.2	2.1	**	6.7	5.7	**
	0.5	3.3	3.3	NS	2.7	2.0	**	6.0	5.3	**
	1	3.4	3.2	NS	2.5	2.1	**	5.9	5.3	**
	2	2.6	3.0	**	2.2	1.8	**	4.8	4.8	NS
	4	2.6	2.7	NS	2.1	1.7	**	4.7	4.4	NS
‡SE		0.02			0.02			0.02		
§CV(%)		9			10			5		

** significant at P ≤0.05, †NS: not significant at P ≤0.05

‡ SE = standard error of the mean

§CV = coefficient of variation

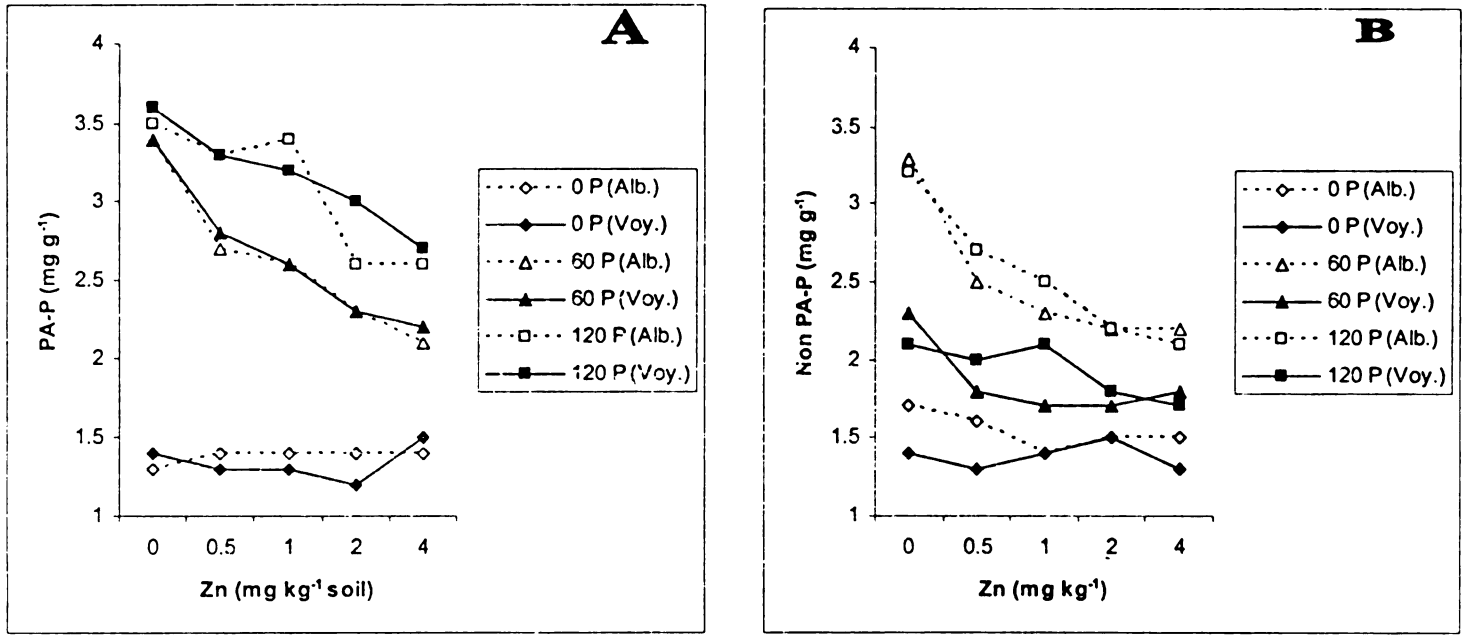

Figure 4. Comparison of the concentrations of phosphorus in phytic acid [PA-P] and non- phytic acid [non PA-P] forms in seed of Voyager (Voy) and Albion (Alb) navy bean cultivars grown under three levels of P (0, 60, and 120mg P kg^{-1}) and five levels of Zn (0, 0.5, 1, 2, and 4 mg Zn kg^{-1}) fertilizer. Fig. 4A shows the responses for the two cultivars for seed phosphorus in the phytic acid [PA-P] form. Fig. 4B shows the responses for the two cultivars for seed P in the non phytic acid [non PA-P] form.

Table 2. Means for phytic acid phosphorus (PA-P) content per experimental unit (seed per 3 plants), non-phytic acid phosphorus (non PA-P) content per experimental unit and total phosphorus (P) content per experimental unit in Voyager and Albion navy bean cultivars grown under three levels of P and five levels of Zn fertilizer.

Soil treatment		PA-P Content		Sig. of	Non PA-P Content		Sig. of	Total P Content		Sig. of
P	Zn	Albion(a)	Voyager(b)	a-b at	Albion(c)	Voyager(d)	c-d at	Albion(e)	Voyager(f)	e-f at
mg kg^{-1}		——mg 3 plants^{-1}——		P ≤0.05†	——mg 3 plants^{-1}——		P ≤0.05†	——mg 3 plants^{-1}——		P ≤0.05†
0	0	19.1	24.6	NS	23.6	24.4	NS	42.7	49.0	NS
	0.5	19.9	21.8	NS	22.5	21.2	NS	42.4	43.0	NS
	1	23.1	20.8	NS	22.6	22.3	NS	45.7	43.2	NS
	2	19.2	18.9	NS	19.4	22.1	NS	38.6	41.0	NS
	4	20.4	22.0	NS	20.3	20.0	NS	40.7	42.0	NS
60	0	40.4	78.0	**	38.0	37.4	NS	78.5	115.3	**
	0.5	60.3	70.0	**	56.5	46.4	**	116.8	116.4	NS
	1	58.9	67.8	**	53.6	44.3	**	112.4	112.2	NS
	2	60.1	63.0	NS	54.8	43.9	**	114.9	106.8	NS
	4	54.5	56.8	NS	55.3	43.6	**	109.8	100.4	NS
120	0	21.4	89.2	**	18.0	51.3	**	39.3	140.5	**
	0.5	64.5	86.0	**	50.7	52.1	NS	115.2	138.1	**
	1	73.4	85.3	**	52.1	56.7	NS	125.2	142.0	**
	2	74.9	89.8	**	61.0	54.5	NS	135.9	144.3	NS
	4	76.5	81.6	NS	60.0	51.4	**	136.4	133.0	NS
‡SE			2.85			2.44			3.94	
§CV(%)			11			12			9	

** significant at P ≤0.05, †NS: not significant at P ≤0.05,
‡SE = standard error of the mean
§CV = coefficient of variation

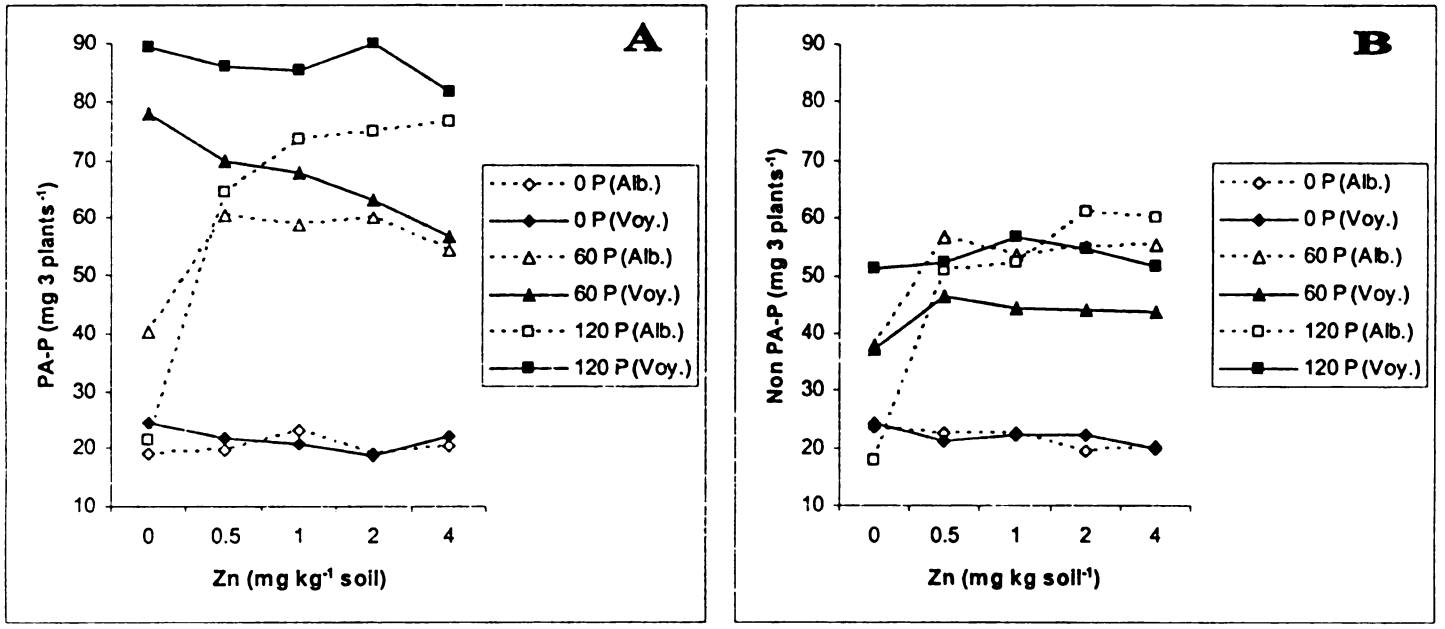

Figure 5. Comparison of the content per experimental unit (seed of 3 plants) of phosphorus in phytic acid (PA-P) and non-phytic acid (non PA-P) forms in seed of Voyager (Voy) and Albion (Alb) navy bean cultivars grown under three levels of P (0, 60, and 120mg P kg^{-1}) and five levels of Zn (0, 0.5, 1, 2, and 4 mg Zn kg^{-1}) fertilizer. Fig. 5A shows the responses for the two cultivars for seed phosphorus in the phytic acid (PA-P). Fig. 5B shows the responses for the two cultivars for seed non PA-P

Seed PA-P and Non PA-P content (Table 2 and Fig. 5) which is the amount of each of the components per experimental unit (seed from 3 plants), showed similar patterns of accumulation as compared to PA-P and non PA-P concentration, respectively (Table 1 and Figure 4). One notable exception was the response by 'Albion' at 60 and 120 mg kg^{-1} P fertilizer treatments to the addition of 0.5 mg kg^{-1} Zn fertilizer treatment. The large increase in PA-P and Non PA-P content resulted from a corresponding increase in seed yield (Fig. 6) at these P levels with the addition of 0.5 mg kg^{-1} soil Zn. The concentration of seed PA-P and Non PA-P did not increase because of the dilution effect caused by the increased seed yield.

The various concentration responses noted in the seed when P and Zn fertilizer levels were increased provided a basis for making inferences from the data. First, the levels of seed [Non PA-P] were more tightly controlled than seed [PA-P] such that when excess P was added to the soil, the P most likely was translocated into seed PA rather than in some other form of P. This was especially true when the levels of P fertilizer were above 60 mg kg^{-1} soil. The data upon which this assumption is based is limited to measurements of seed P. Previous studies in common bean where the effects of P fertilizer on P in other plant parts were investigated also support this assumption. For example, Lynch et al., (1991) indicated that as P fertilizer increased leaf [P] also increased. As P available to the roots increased so did the amount of P translocated to the shoots (ranging from 20 to 80% of P in roots). On the other hand, remobilization of Zn and P to the seed from shoots and leaves remained constant under low or high P fertilizer conditions at 60 to 67% of the total Zn and P in the shoots and stems (Snapp and Lynch, 1996). The results of the current study

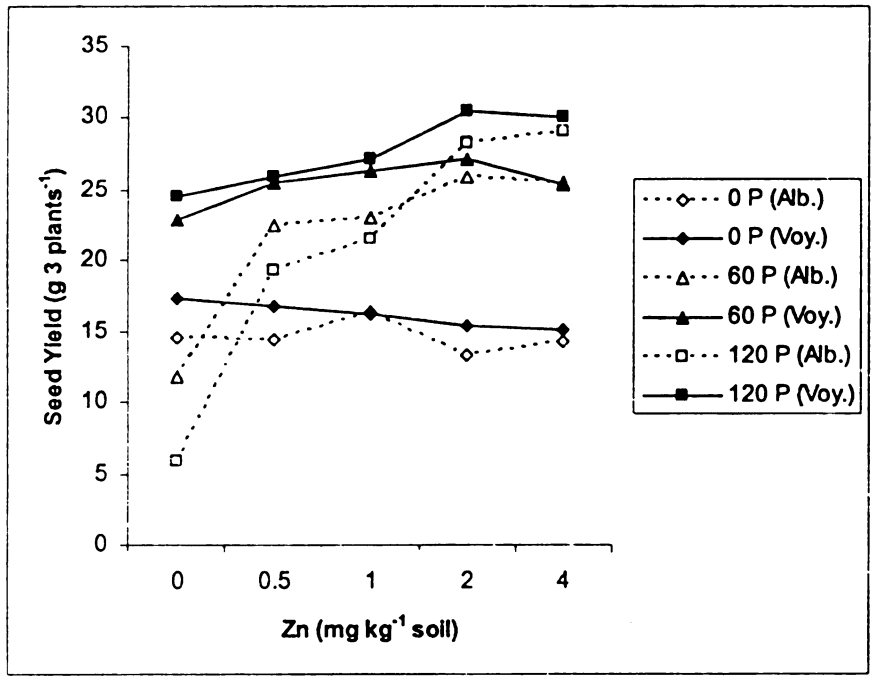

Figure 6. Seed yield per experimental unit (3 plants) of Voyager (Voy) and Albion (Alb) navy bean cultivars grown under three levels of P (0, 60, and 120mg P kg^{-1}) and five levels of Zn (0, 0.5, 1, 2, and 4 mg Zn kg^{-1}) fertilizer.

suggested that, in general, as P fertilizer increases a higher proportion is expected to accumulate in the seeds because of the increased translocation from the roots to the shoots. The work of Snapp and Lynch (1996) support this suggestion. The differences in seed [PA-P] and [Non PA-P] noted at each treatment level could also be due to 'Voyager' and 'Albion' sharing common alleles for the genes that control the regulation of PA synthesis in the seed. However, the levels of at least one of the forms of non PA-P in the seed may be regulated differently in 'Voyager' as compared to 'Albion'. Non PA-P is found by subtracting PA-P from total P and includes components of molecules such as DNA, RNA, sugars, phospholipids, phosphate esters (other than phytic acid) and free inorganic phosphate.

Yield differences between 'Albion' and 'Voyager' in response to Zn fertilizer treatments are shown in Figure 6. At the fertilizer treatments 0 mg kg^{-1} soil Zn, 120 mg kg^{-1}soil P and 0 mg kg^{-1}soil Zn, 60 mg kg^{-1}soil P, 'Albion' showed a markedly reduced seed yield compared to 'Voyager' at the same fertilizer levels. The yield of 'Albion' was significantly increased by added Zn fertilizer, and although 'Voyager' also showed a yield increase in response to added Zn, it was less dramatic than the response of 'Albion'. For example, at the 120 mg kg^{-1}soil P, 0 mg kg^{-1}soil Zn treatment, the seed yield of 'Albion' was 50% lower than at the 60 P mg kg^{-1}soil, 0 mg kg^{-1}soil Zn treatment. The seed yield for 'Albion' at 0 mg kg^{-1}soil P, 0 mg kg^{-1}soil Zn was two to three times higher than at the 60 mg kg^{-1}soil P, 0 mg kg^{-1}soil Zn and 120mg kg^{-1}soil P, 0 mg kg^{-1} soil Zn treatments, respectively. These data indicated that a plant response to phosphorus was a key factor in differentiating 'Voyager' (Zn efficient) from 'Albion' (Zn inefficient).

In addition to differences in yield, 'Albion' and 'Voyager' also showed differences in levels of total P accumulation. 'Albion' had significantly higher levels of seed P than 'Voyager' at the 60 mg kg^{-1}soil P, under all levels of Zn fertilizer, and at 120 mg kg^{-1}soil P under 0, 0.5, and 1 mg kg^{-1}soil Zn treatments (Table 1). Moraghan and Grafton (1999) saw similar higher levels of P in the seed of Zn inefficient navy bean cultivars as compared to efficient cultivars at low levels of soil Zn. However, Moraghan and Grafton (1999) did not look at the effect of different P fertilizer levels on the concentration of P in the seed of the cultivars. For the current study, there was different P fertilizer treatments in addition to different Zn fertilizer treatments. Based on these data, phosphorus induced Zn deficiency may be the cause of the Zn inefficiency characteristic of 'Albion'. One may speculate that the gene(s) that control Zn efficiency in navy bean also may be involved in P regulation in the seed or the mechanism of Zn efficiency may be related to the plant's ability to exclude P from the shoots and seeds.

Zn efficiency in bean not only influenced seed yield in low Zn soils, but also seed [Zn] (Table 3). At 0 mg kg^{-1} soil P, at all levels of Zn fertilizer, 'Voyager' had, on average, 17% higher seed [Zn] than 'Albion'. Also, at 60 mg kg^{-1} soil P and 120 mg kg^{-1} soil P at 2, and 4μg kg^{-1}Zn fertilizer, seed [Zn] in 'Voyager' exceeded the seed [Zn] of 'Albion' by an average of 23%. In this study, 'Voyager' generally had higher seed [Zn] than 'Albion' at low soil P levels and at intermediate to high soil P levels when Zn levels in the soil were high (Table 3).

The results of this study may provide insight as to whether navy beans with a high concentration of seed zinc have an improved Zn nutritional value over beans

Table 3. Means for seed yield, seed zinc concentration [Zn] and zinc content per experimental unit (Zn) (seed of three plants) in Voyager and Albion navy bean cultivars grown under three levels of P and five levels of Zn fertilizer.

Treatment		Seed Yield		Sig. of	Seed Zinc		Sig. of	Seed Zinc		Sig. of
P	Zn	Albion(a)	Voyager(b)	a-b at	Albion(c)	Voyager(d)	c-d at	Albion(e)	Voyager(f)	e-f at
mg kg^{-1} (soil)		g 3 plants^{-1}		P ≤ 0.05†	μg g^{-1}		P ≤ 0.05	μg 3 plants^{-1}		P ≤ 0.05
0	0	14.6	17.4	**	22.9	28.5	**	334	494	**
	0.5	14.5	16.8	NS	28.2	33.2	**	410	558	**
	1	16.4	16.2	NS	29.5	38.7	**	483	628	**
	2	13.4	15.5	NS	33.9	40.0	**	457	623	**
	4	14.3	15.1	NS	37.8	42.7	**	539	646	**
60	0	11.9	22.9	**	18.4	16.4	NS	218	373	**
	0.5	22.5	25.5	**	18.0	19.7	NS	405	502	**
	1	23.0	26.3	NS	18.5	22.4	**	426	587	**
	2	25.9	27.1	NS	20.4	25.7	**	527	695	**
	4	25.5	25.4	NS	23.6	34.1	**	603	867	**
120	0	5.9	24.5	**	14.6	16.1	NS	96	395	**
	0.5	19.3	25.9	**	15.8	15.9	NS	305	412	**
	1	21.5	27.1	**	17.8	18.0	NS	382	486	**
	2	28.3	30.4	NS	16.6	19.4	**	470	588	**
	4	29.1	30.0	NS	19.0	24.0	**	553	720	**
‡SE			0.96			0.71			29.32	
§CV(%)			9			6			12	

** significant at P ≤0.05, †NS: not significant at P ≤0.05,
‡SE = standard error of the mean
§CV = coefficient of variation

with a low concentration of Zn. Before this question can be answered studies should be conducted to ascertain whether the results of greenhouse pot studies on seed [Zn] correlate highly with field experiments. Second, culinary studies are necessary to establish whether Zn levels of Zn efficient and inefficient cultivars are significantly different after beans are prepared for consumption. Finally, Zn feeding studies involving Zn and PA are needed to determine what levels of seed [Zn] in navy bean can improve human nutrition.

In dry bean, one must give consideration to [PA] in attempts to improve the micronutrient density of this crop. Once consumed in human foods PA binds to minerals in the intestinal tract to form mixed salts that are largely excreted (Raboy et al., 2001). Hence, Zn bioavailability in humans whose diets consist mainly of cereals and legumes is dependant not only on Zn intake but also the intake of PA.

In the present study, 'Albion' and 'Voyager' did not differ in their amount of PA-P, but they differed in the amount of non PA-P and total P (Table A1). Hence, one can conclude that 'Albion' and 'Voyager' differed significantly in the % of total P stored as phytic acid at the 60 and 120 mg kg^{-1} P fertilizer treatment level (Table 4). 'Voyager' had a higher % of total P stored as PA than 'Albion' because of the differences in the non PA-P fractions. The differences between 'Voyager' and 'Albion' noted in this study for PA-P and Non PA-P suggests that selecting plants with low phytic acid or low % of total P stored as PA per se may not be the most effective way to reduce seed phytic acid. Raboy et al. (1990) suggested the use of increased inorganic P levels in the seed as a selection criterion because total P and PA-P are strongly correlated. By selecting for reduced PA-P one may also decrease

Table 4. The percent of phosphorus (P) in the phytic acid (PA) form in seed of Voyager and Albion navy bean cultivars grown under three levels of phosphorus and five levels of zinc fertilizer.

Soil treatment		P in PA form		Sig. of
P	Zn	Albion(a)	Voyager(b)	a–b at
mg kg^{-1}		----------%----------		P ≤0.05†,**
0	0	44.8	50.3	NS
	0.5	47.0	50.8	NS
	1	50.5	48.2	NS
	2	49.6	46.6	NS
	4	50.0	53.2	NS
60	0	51.3	67.6	**
	0.5	51.6	60.2	**
	1	52.5	60.4	**
	2	52.3	59.1	**
	4	49.4	56.4	**
120	0	52.6	63.4	**
	0.5	55.8	62.4	**
	1	58.5	60.1	NS
	2	55.0	62.2	**
	4	56.1	61.5	NS
‡SE		2.24		
§CV(%)		8		

** significant at P ≤0.05, †NS: not significant at P ≤0.05
‡ SE = standard error of the mean
§CV = coefficient of variation

Table 5. The regression relationship (R^2) between three phosphorus (P) treatments added to the soil and the concentrations of phosphorus in phytic acid (PA-P) and non-phytic acid (non PA-P) forms in seed of two navy bean cultivars.

	P added to soil (mg P kg^{-1})			
Cultivar	0	60	120	R^2
	---------PA-P (mg g^{-1})---------			
Albion	1.39	2.62	3.11	0.74
Voyager	1.35	2.67	3.16	0.75
	------- Non PA-P (mg g^{-1})------			
Albion	1.49	2.48	2.5	0.47
Voyager	1.36	1.69	1.94	0.57

Table 6. Multiple linear regression for the effect of cultivar and P and Zn added to the soil on phytic acid phosphorus (Pa-P), non-phytic acid phosphorus (non Pa-P), and zinc (Zn) in the seed of two navy bean cultivars.

	Pa-P (Y1)		Non Pa-P (Y2)		Zn (Y3)	
Overall R^2	0.81		0.64		0.85	
Correlation	|r|	p value	|r|	p value	|r|	p value
Cultivar (X1)	0.01	NS	-0.46	<.0001	0.24	0.008
P fertilizer (X2)	0.86	<.0001	0.60	<.0001	-0.79	<.0001
Zn fertilizer (X3)	-0.26	0.0045	-0.26	0.0042	0.43	<.0001

the viability of the seed along with impairing its nutritional value. Seedling emergence and dry weight accumulation are greater in *P. vulgaris* seed with high levels of P as compared to seed with low levels of P (Teixeira et al., 1999). In the current study, there was a strong linear relationship between seed [PA-P] and P fertilizer level (r= 0.86) (Table 5 and Table 6) therefore by selecting for reduced levels of PA, one would also be selecting for reduced total seed P. Phosphorus fertilizer level was also correlated with seed [non PA-P], but the correlation was not as strong as the correlation between P fertilizer level and seed [PA-P]. Based on the results of the current study, the screening of germplasm based on Pi may prove fruitful to lower [PA] in dry bean because a common phenotype seen in low phytic acid mutants in other crops is an increase in Pi corresponding to the decrease in [PA-P] (Raboy, 2001). Selecting germplasm for increased seed [Zn] appears to be a promising method to increase the zinc density of the seed, which may improve zinc bioavailability in terms of human nutrition. Not only was significant genotypic variability found for seed [Zn], but seed [Zn] and [PA-P] were negatively correlated. Figures 7, A1 and A2 show that seed [PA-P] and [Zn] are negatively correlated, which indicates selection based solely on seed [Zn] will not cause an increase in seed [PA-P].

CONCLUSIONS

The differences in seed yield, [Zn], and [P] in 'Voyager' and 'Albion' indicated that the zinc inefficiency trait may be caused by P induced Zn deficiency and that the signs of P induced Zn deficiency documented in roots and shoots of certain bean cultivars is also seen in the seed of Albion.

'Voyager' (Zn efficient) had higher seed [Zn] than 'Albion' (Zn inefficient) at various P and Zn fertilizer levels. Intrinsic differences between the cultivars for seed zinc status may be useful to improve the zinc density of the seed to make more zinc available in diets. 'Voyager' and 'Albion' did not differ in their amount of PA-P, but seed [Zn] and [PA-P] were negatively correlated, indicating that a breeding strategy to increase seed Zn should not cause a simultaneous increase in seed PA-P.

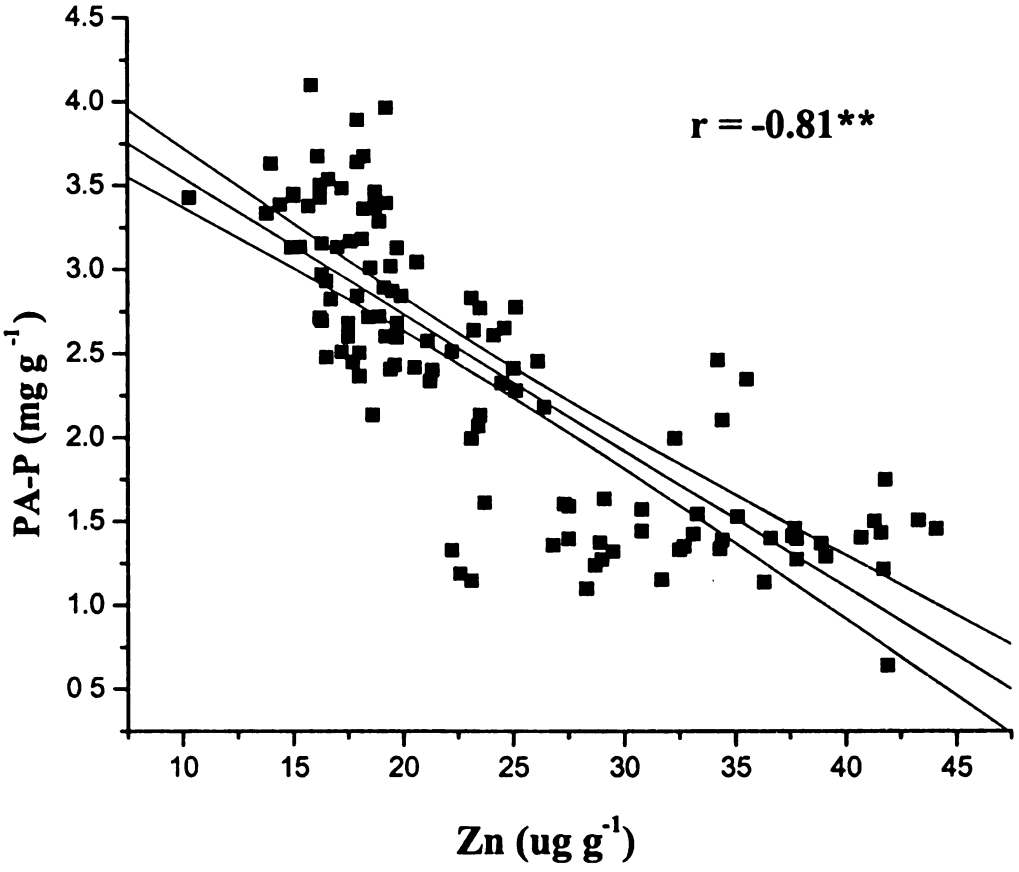

Figure 7. Correlations between the concentration of zinc [Zn] and the concentration of phosphorus in the phytic acid form [PA-P] in the seed of Albion and Voyager navy beans grown under three levels of P (0, 60, and 120mg P kg^{-1}) and five levels of Zn (0, 0.5, 1, 2, and 4 mg Zn kg^{-1}) fertilizer.

** significant at P $\leq$0.05

CHAPTER 2: INHERITANCE OF SEED PHYTIC ACID PHOSPHORUS AND ZINC LEVELS IN THE SEED OF NAVY BEAN

INTRODUCTION

Improving yield over a range of environments is generally the major goal of dry bean breeding programs. However, bean breeders should not neglect food quality improvements. Food quality in dry bean includes characteristics that have a direct impact on human nutrition and those related to preparation for eating.

Breeding for improved nutritional quality is an endeavor that requires much consideration. Enhancement of vitamins and minerals and reduction of antinutrients in edible plant parts will not likely improve yield, and may actually decrease it (Bliss, 1999). Except for economically important plant parts with enhanced carotene or lycopene content, the appearance of nutritionally enhanced plants generally are not noticeable to breeders, growers, or consumers. The benefits from improved nutritional quality in a crop will only be realized by a population that has a deficiency in the diet for the given vitamin or mineral. Do human populations exist that can benefit from plant modification for increased nutritional quality? If one considers the zinc status in humans, an estimated 49% of the world population is at risk for low zinc intake (Brown et al., 2001). The areas of the world where people are most at risk are South and Southeast Asia, Sub-Saharan Africa, and Latin America and the Caribbean. In these regions, only 15 to 25% of the zinc is from animal sources as compared to developed nations, where more than half the zinc

comes from animal sources. In many developing countries cereals and legumes are staples in the diet. Phytic acid is an important antinutrient in these crops. The high phytate: zinc molar ratios of plant based sources of zinc such as grains and legumes as compared to animal sources of zinc is a major factor in determining the risk of zinc deficiency (Frossard et al., 2000).

Zinc deficiency symptoms in humans include impairment of physical growth, diarrhea, increased incidence of pneumonia and other upper respiratory infections, decreased neurophysical performance, and impaired immune function (Hambidge, 2000). The diverse symptoms of zinc deficiency can be attributed to its central role in many physiological processes. Zinc is essential for over 300 enzymes, hormones, and structural proteins (Penny, 1998). Studies in Vietnam, Mexico, Guatemala, India, Jamaica, Indonesia, Papua New Guinea, and Peru have demonstrated the reduction of diarrhea and pneumonia associated with giving children daily zinc supplementation (Penny, 1998). Supplementation and fortification are traditional solutions that have been applied to nutrient deficiencies, and may also work to decrease zinc deficiency in target populations (Ruel and Bouis, 1998). But, these are not sustainable strategies and require a change in consumer behavior (Rengel et al., 1999). On the other hand, increasing the bioavailable zinc in the diet through plant breeding, offers a sustainable strategy that does not require a change in eating habits.

Plant breeding to meet consumer recommended dietary allowances (RDA) for a given nutrient is a time consuming and costly process. Therefore a survey should be conducted to determine the need, impact, and acceptability of a nutritionally enhanced crop (Bliss and Quebedeaux, 1988). To maximize time and resources, the

breeder must possess some knowledge of the range of variability and the nature of gene action of the trait.

Before a plant-breeding program is initiated to improve a particular trait, the breeder must have knowledge of gene action involved in trait expression and the degree to which genes are influenced by environmental fluctuation. Knowledge of the type of gene action for a trait aids the breeder in ascertaining which breeding procedure will efficiently improve the performance of a trait. Accordingly, a study was conducted with three objectives: 1) ascertain the inheritance of zinc and phytic acid levels in navy bean. 2) determine the broad and narrow sense heritability of phytic acid and zinc levels, and 3) discuss the results in terms of breeding strategies to improve zinc content in dry bean seeds.

MATERIALS AND METHODS

'Voyager' a zinc efficient navy bean and 'Albion' a zinc inefficient navy bean were chosen for the study based on their differences in seed zinc concentration. A zinc efficient genotype has normal seed yield under low soil zinc conditions, whereas a zinc inefficient genotype has a reduced yield under low zinc soil levels that is increased by the addition of zinc fertilizer. In 1998, seed of 'Voyager' and 'Albion' was planted in a greenhouse in a soil-less mixture (Sunshine Mix, Fison's Inc.) fertilized with 12-12-12 time-release fertilizer and watered daily. Upon flowering, 'Voyager' and 'Albion' were cross-fertilized to produce F_1 seed. In 1999, F_1 plants were hybridized to each parent to produce backcross generations and F_1 plants were allowed to self-pollinate to produce F_2 seed. Seed was harvested from each plant individually and seed of each F_1 plant was kept separate from F_2 seed of other plants.

In 1999 seed of the following generations: parent 1 ('Albion'), parent 2 ('Voyager'), F_1, F_2, BC_1P_1, and BC_1P_2 were hand-sown at Erie, ND. The soil type was an Eckman (coarse-silty, mixed, superactive, frigid Calcic Hapludolls) with pH of 5.8 and 2.4 mg kg^{-1} DTPA-Zn, 84 mg kg^{-1} DTPA-Fe and 21 mg kg^{-1} $NaHCO_3$-P. Thirty seeds were distributed uniformly in 1.5-m rows, spaced 0.76 m apart. One row was planted for each parent and the F_1, BC_1P_1, and BC_1P_2 , and eight rows of F_2 seed were planted. Population size evaluated was based on seed availability. Fertilizer and herbicide applications and common cultural practices followed recommended practices for dry bean production in the northern Great Plains. Plots were hand weeded as necessary. Plants were harvested individually by hand and kept separate by placing each plant in a paper bag. Pods were removed from plants and seed threshed from pods in the greenhouse by hand to avoid any contamination with Zn containing appliances.

The pods were threshed by hand and a sub-sample of seed from each plant was analyzed for total phosphorus, phytic acid, and Zn. Total P was measured by a molybdenum blue procedure and zinc was measured by atomic absorption. For each analysis, 0.5 g of seed was ashed in a muffle furnace at 500°C for 6 hours. Next, 25ml of 3N HNO3 was added to the seeds. After one hour the mixture was filtered with paper filters to remove undissolved particles. These samples were directly analyzed for zinc concentration by atomic adsorption spectroscopy. For P analysis, the samples were diluted 10:1 v:v in 0.3N NaOH. Following the dilution, they were diluted 15;1 v:v with 2.5N sulfuric acid containing 0.006% ammonium molybdate,

0.0002% antimony potassium tartrate, and 0.005% l-ascorbic acid. The sample absorbance was read on a Brinkmann dipping probe with an 880nm filter.

An additional subsample of seed was freeze dried, ground, and analyzed for phytic acid. Sample preparation for phytic acid [PA] analysis in bean seed was conducted following the procedure of Lehrfeld (1980). Phytic acid was extracted from the samples by the addition of 3ml 0.5M HCl (trace element grade) to 0.100 g of sample. The acidified samples were mechanically agitated for 2 hours at 21°C. Samples were centrifuged at 12,000g for 15 minutes. The supernant was collected and diluted 1:5 (v/v) with distilled, deionized water. The diluted sample (15 ml total) was passed through a Bond Elut strong anion exchange column (Varian, Walnut Creek, CA) for purification. The column was washed once with 10 ml 0.05 M HCl, and phytic acid was eluted with 3ml 2M HCl. The eluted fraction containing PA was air dried and dissolved in 5mM sodium acetate. The dissolved sample was filtered through a 2μm filter. Phytic acid levels in each sample were quantified by high performance liquid chromatography with a refractive index detector. The column used for analysis was a Waters Symmetry C18 column (3.9mm x 150mm) (Waters, Milford, MA) heated to a temperature of 40°C. Sodium acetate (5mM) was used as the solvent at a flow rate of 1.4 ml min^{-1}. Phytic acid dodecasodium salt from corn (*Zea. mays* L.) (Sigma, St. Louis, MO) was used as a standard to determine PA concentration.

Based on these data, the following characters were analyzed for variability: total phosphorus (P), phytic acid-phosphorus (PA-P), non-phytic acid-phosphorus (non PA-P), percent phosphorus as phytic acid (%PA-P), and zinc (Zn). PA-P was

determined by dividing the concentration of phytic acid by 3.55 (Lott at al., 2000) and non PA-P was determined by subtracting PA-P from total P.

Chi-square analyses were conducted for frequency distributions of seed Zn in the F_2 and $BC_1P_{1,2}$ generations to test the goodness of fit of the data to hypothesized genetic ratios. Seed samples were classified into two groups, low zinc and high zinc. Discriminant analysis was the statistical procedure used to classify each plant in the F_2 and backcross generations into either high or low seed [Zn]. In this procedure, the pooled standard deviation of Voyager and Albion was estimated. The cutoff value between the high and low Zn classes was determined by subtracting the mean of Albion from the mean [Zn] of Voyager. That number was divided by the pooled variance. The resulting number was multiplied by the mean of Albion plus the mean of Voyager and divided by two. Any value above the resulting number was categorized as high seed [Zn], and any sample with a value below the number was categorized as low seed [Zn].

A mixed effects model (PROC MIXED in SAS) was used to determine significant variation among means of the six generations for each of the five seed characteristics (seed total P, PA-P, non PA-P, %P as PA, and Zn) of interest. In this model, replications were considered random effects, and generations were considered fixed effects. Variance was partitioned in a generation means analysis model according to the model: $Y = m + (\alpha)[d] + (ß)[h] + (\alpha^2)[i] + (2\alpha ß)[j] + (ß^2)[l]$ where Y was the observed mean of a generation (Holthaus et al., 1996).

Weighted least squares regression was used to fit six generations to six variables beginning with the midparent. Each generation was weighted by the inverse of the

variance of its mean (Mather and Jinks, 1971). The six variables used in the model to describe the phenotype were: midparent value (m), additive effects [d], dominance effects [h], additive x additive interactions [i], additive x dominant interactions [j], and dominance x dominance interactions [l]. The model parameters were estimated from the means of each generation and genetic coefficients (Table 7). Relationships among the six generations used to establish gene effect estimates for generation means analysis (Gamble, 1962) were:

$$
\begin{array}{lcl}
m & = & F_2 \\
[d] & = & P_1F_1 - P_2F_1 \\
[h] & = & -\tfrac{1}{2}P_1 - \tfrac{1}{2}P_2 + F_1 - 4F_2 + 2P_1F_1 + 2P_2F_1 \\
[i] & = & -4F_2 + 2P_1F_1 + 2P_2F_1 \\
[j] & = & -\tfrac{1}{2}P_1 + \tfrac{1}{2}P_2 + P_1F_1 - P_2F_1 \\
[l] & = & P_1 + P_2 + 2F_1 + 4F_2 - 4P_1F_1 - 4P_2F_1
\end{array}
$$

From the above estimates, expected means were found and a chi square test was used to determine if the data fit an additive/ dominance model which only included m, [d], and [h] or an epistasis model which takes all six gene effects into consideration. T-tests were used to determine which of the genetic estimates were significant. These analyses were conducted with the aid of a spreadsheet developed by Ng (1990).

Estimates of broad sense and narrow sense heritability were calculated for seed zinc concentration by using the variance of the parent, F_1, F_2, and backcross generations to estimate phenotypic (V_P), environmental (V_E), total genetic (V_G), additive genetic (V_A), and dominance genetic variances (V_D). Where:

$V_P = V_{F2}$
$V_E = \frac{1}{4}(V_{P1}) + \frac{1}{4}(V_{P2}) + \frac{1}{2}(V_{F1})$
$V_G = V_{F2} - V_E$
$V_A = 2(V_{F2}) - V_{BC1P1} - V_{BC1P2}$
$V_D = V_{BC1P1} + V_{BC1P2} - V_{F2} - V_E$

Table 7. Coefficients of gene effects in generation means analysis.

Generation	Gene effects					
	m	[d]	[h]	[i]	[j]	[l]
P_1	1	1	0	1	0	0
P_2	1	-1	0	1	0	0
F_1	1	0	1	0	0	1
F_2	1	0	½	0	0	¼
BC_1P_1	1	½	½	¼	¼	¼
BC_1P_2	1	-½	½	¼	-¼	¼

m: midparent, [d]: additive [h]: dominance [i]: additive x additive
[j]: additive x dominance [l]: dominance x dominance (Holthaus et al., 1996).

Broad sense heritability = $h^2_b = (V_A + V_D)/V_{F2}$ where $V_A + V_D$ represent the genetic variance of F_2 (Allard, 1960). Narrow sense heritability = $h^2_n = V_A/V_{F2}$ (Warner, 1952).

RESULTS AND DISCUSSION

Except for BCP_2, there were no differences among generations for total seed phosphorus (Table 8 and Fig 8). The six generations showed various differences among generations for phytic acid phosphorus, non-phytic acid phosphorus, and % of total phosphorus as phytic acid (Table 8). The lack of variability for total seed P precluded performing a generation means analysis in the 'Albion' x 'Voyager' derived population.

Seed Phytic Acid Phosphorus Concentration:

The mean PA-P concentration of Albion and Voyager was 1.7 mg g^{-1} and 2.0 mg g^{-1} respectively (Table 8). Since these values were not significantly different from each other, the F_2 and BC generation data were not able to be categorized into classes of high and low phytic acid phosphorus. However, generation means analysis provided data to make a few genetic inferences. First, based on linear contrasts, the mean of the F_1 generation (2.19) deviated significantly from the mid-parent value (Figure 9). A significant deviation of the F_1 hybrid from the mid-parent indicates that dominance was a property of the genetic system influencing phytic acid phosphorus content in seeds. The broad sense heritability for phytic acid phosphorus was 0.17 (Table 11). This low value indicated that environmental effects greatly overshadowed genetic effects for this trait, thus selecting for low PA-P will

Table 8. Mean levels of total seed phosphorus (P), the phytic acid (PA-P) and non-phytic acid fractions of phosphorus (non PA-P), the percent of total seed phosphorus in the phytic acid form (%PA-P) and seed zinc (Zn) in two navy bean cultivars and four additional generations established by an initial cross between the cultivars.

Generation	Phosphorus (mg/g)	Phytic acid P (mg/g) [PA-P]	Non phytic acid P (mg/g) [non PA-P]	Percent total P as phytic acid (%)	Zinc (μg/g)
P_1†	4.9 *a*‡	1.7 *a*	3.2 *a*	35 *a*	21.7 *a*
P_2†	4.9 *a*	2.0 *a,b*	2.9 *a,b,d*	42 *a,b*	31.2 *b*
F_1	5.0 *a*	2.2 *b*	2.8 *a,e,f*	45 *b*	28.5*c,d,e*
F_2	4.9 *a*	2.5 *c*	2.5 *b,e,g*	52 *c*	28.5 *c*
BC_{P1}	4.9 *a,c*	2.5 *c*	2.5 *d,f,g*	52 *c*	26.5 *e*
BC_{P2}	4.3 *b,c*	2.8 *d*	1.7 *c*	64 *d*	30.7 *b,d*
F Value	1.40	20.65	5.63	12.53	19.12
P	0.26	<.0001	<.0005	<.0001	<.0001

† P_1 and P_2 are Albion and Voyager, respectively
‡Means the do not share a letter are significantly different from each other (Tukey's test, $P < 0.05$).

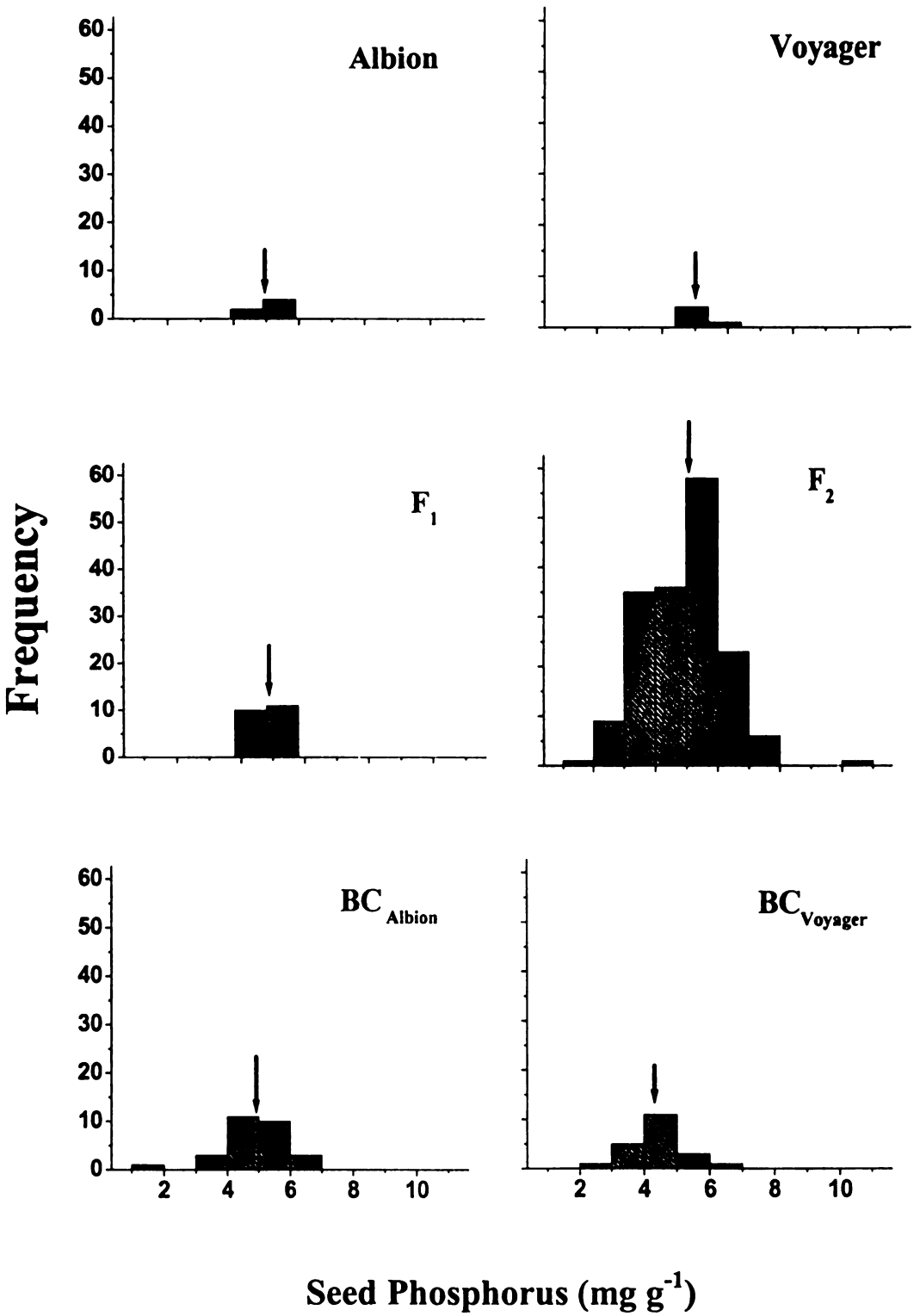

Figure 8. Frequency distribution of phosphorus concentration in Albion and Voyager navy bean seed and four generations derived from Albion x Voyager. The arrows and number in parenthesis indicate the mean phosphorus concentration of each generation.

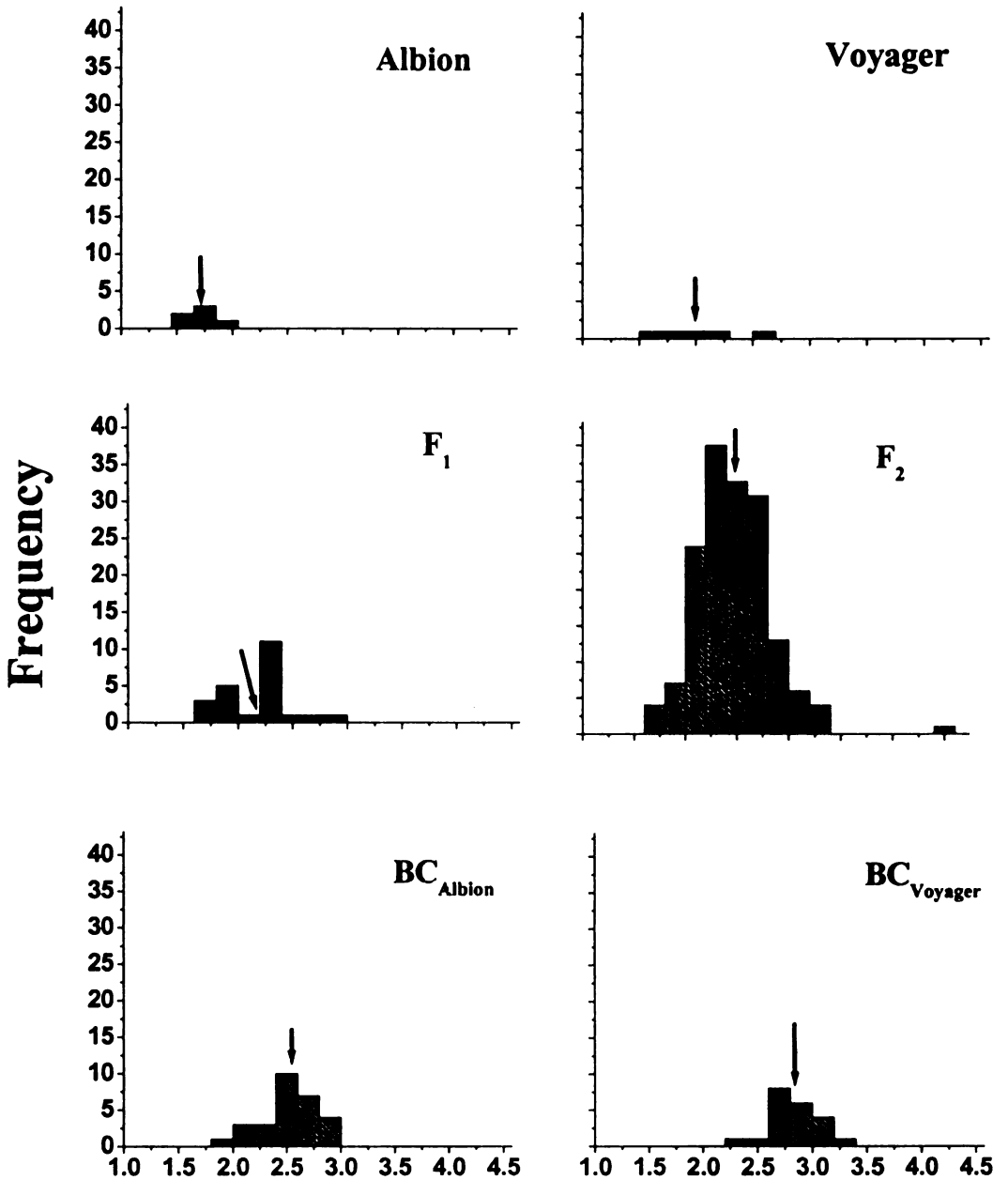

Figure 9. Frequency distribution of phytic acid phosphorus concentration [PA-P] in Albion and Voyager navy bean seed and four generations derived from the intermating of Albion and Voyager. The arrows indicate the mean [PA-P] of each generation.

Table 9. Chi-square test for the adequacy of an additive/dominance generation means model for the concentration of seed phytic acid phosphorus [PA-P], non-phytic acid phosphorus [non PA-P], and zinc [Zn] in a cross between Voyager and Albion navy bean cultivars and four additional generations established by an initial cross between the cultivars.

Seed Trait	df	χ^2
PA-P	3	91.9**
Non PA-P	3	23.4**
Zn	3	4.2

*, ** Significance at the 0.05, 0.01 probability levels, respectively based on χ^2 tests with 6-3 = 3 degrees of freedom. Significance indicated the inadequacy of an additive/dominance model.

Table 10. Estimates and standard error of genetic effects in the generation means analysis model for the concentration of seed phosphorus [P], phytic acid phosphorus [PA-P], non phytic acid phosphorus [non PA-P], percent phytic acid phosphorus [% PA-P] and zinc [Zn] in a cross between Voyager and Albion navy bean cultivars and four additional generations established by an initial cross between the cultivars.

	Genetic Effects					
Seed Trait	m	[d]	[h]	[i]	[j]	[l]
PA-P	1.0**	0.16	4.6**	0.82**	0.26	-3.4**
Non PA-P	4.6**	0.16	-6.8**	-1.6**	1.2	4.9**
Zn	26**	4.7**	8.1	0.7	-1.2	-5.3

*, ** Significance at the 0.05, 0.01 probability levels, respectively based on t-tests with n-1 = 5 degrees of freedom

Table 11. The variance components and heritability estimates of phytic acid phosphorus [PA-P], non-phytic acid phosphorus [non PA-P], total phosphorus [P] and zinc [Zn] concentration in the seed derived from a population developed from a cross between Albion and Voyager navy bean.

	Seed Traits			
Variance Components	Zn	PA-P	Non PA-P	Total P
V_P	31.2	0.131	1.5	1.58
V_E	5.9	0.105	0.46	0.294
V_G	25.3	0.026	1.04	1.29
V_A	25.2	0.14	1.57	1.1
V_D	0.1	-0.114	-0.517	0.196
h^2_b	0.85	0.17	0.70	0.84
h^2_n	0.81	-----	-----	------

be difficult, especially in segregating generations. Narrow sense heritability was unable to be estimated because of the presence of epistasis that would confound the estimate (Table 11). It should also be noted that the additive variance was found to be higher than total genetic variance for [PA-P] and non [PA-P] (Table 11). This discrepancy is due to differences is methods of calculation. Based on the generation means analysis of the 'Albion' x 'Voyager' cross, breeding for decreased levels of phytic acid phosphorus will be difficult because of the limited variability and the low heritability. Interestingly, the percent of total P in the phytic acid form was higher in 'Voyager' (42%) than in 'Albion' (35%) but the non PA-P was lower in 'Voyager' (2.9 mg g^{-1}) than 'Albion' (3.2 mg g^{-1}) (Table 8) suggesting that PA-P and non PA-P may be under separate genetic control in the seed.

Seed Non Phytic Acid Phosphorus Concentration:

Differences between 'Albion' and 'Voyager' for levels of non PA-P (Table 8) were minimal. The mid-parent value (3.1 mg g^{-1}) was significantly higher than the F_1 value (2.8 mg g^{-1}) (Figure 10) indicating that low levels of non PA-P is dominant to high levels of non PA-P. Generation means analysis revealed that dominance, additive x additive, and dominance x dominance effects contributed significantly to the inheritance of non phytic acid phosphorus content in this cross (Table 9). The broad sense heritability $h^2_{(B)}$ estimate for non PA-P was 0.7 (Table 11). The estimate of $h^2_{(B)}$ may be meaningless for this trait in this cross because the F_1, F_2 and BC generation means were lower than the mean of either parent. Moreover, the difference between the two parents for non PA-P between the two parents was small,

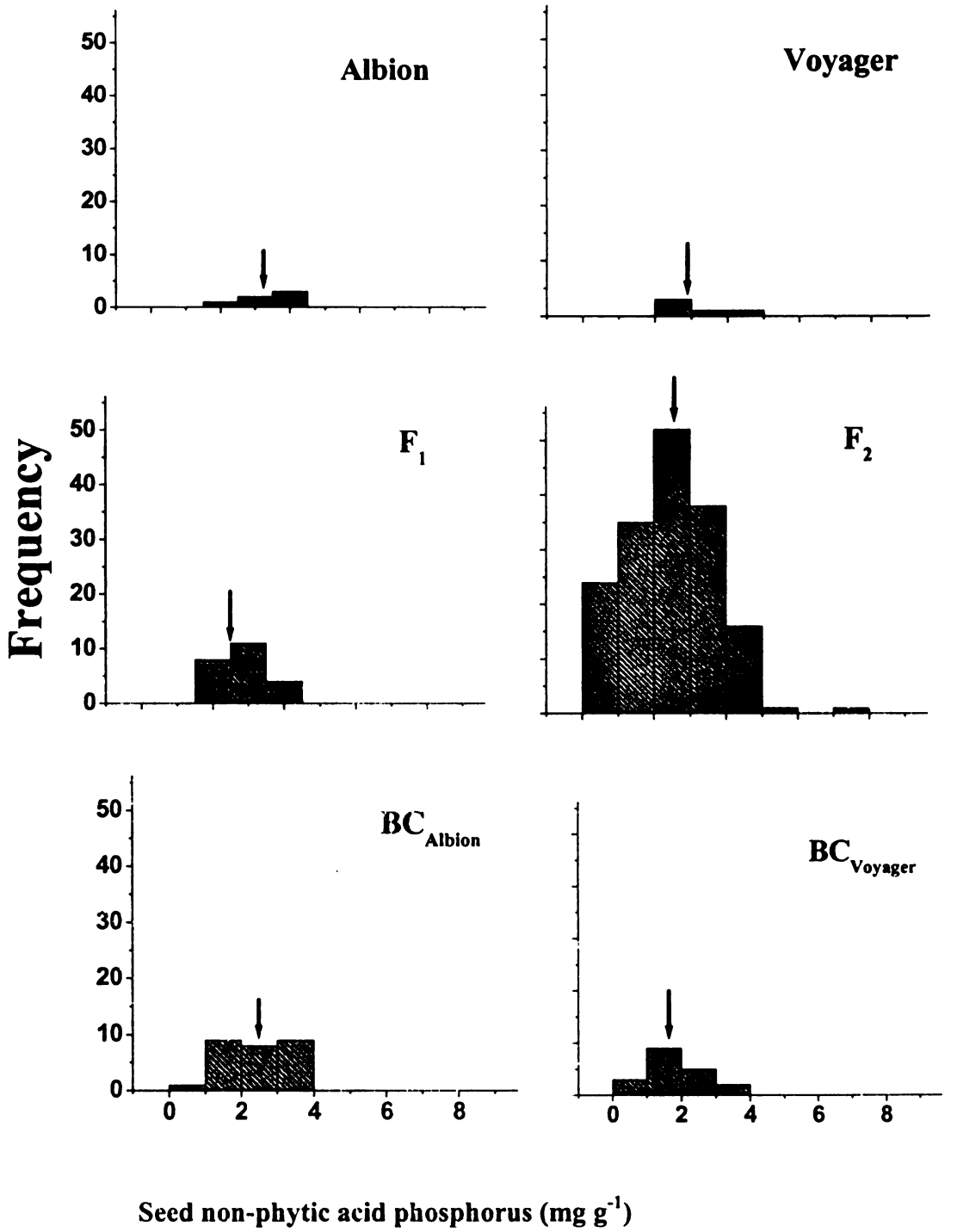

Figure 10. Frequency distribution of the non-phytic acid phosphorus concentration [non PA-P] in Albion and Voyager navy bean seed and four generations derived from the intermating of Albion and Voyager. The arrows indicate the mean [non PA-P] of each generation.

3.2 mg g^{-1} and 2.9 mg g^{-1} for 'Albion' and 'Voyager', respectively (Table 8, Figure 10). Parents that differ little in respect to the character examined cannot generate large additive or genetic variances in their progenies (Simmonds, 1979).

Seed Zinc Concentration:

The zinc inefficient navy bean parent, 'Albion' had a lower seed zinc concentration than 'Voyager', the zinc efficient parent (Table 8). Seed from plants of the F_2 generation derived from Albion x Voyager were scored for high or low seed zinc concentration. The F_2 population consisted of 117 high zinc plants and 51 low Zn plants, which fit a 3:1 ratio based on chi square analysis. The frequency distribution of seed [Zn] in the F2 (Figure 11) was not normally distributed ($p = 0.076$), which also supported a single dominant gene model for the seed [Zn] trait. The BCP_1 consisted of 15 high Zn plants and 14 low Zn plants, which fits a 1:1 ratio based on chi square analysis. The 1:1 ratio in a backcross generation supports the single gene model of inheritance. The BCP_2 consisted of 20 high Zn plants and 1 low Zn plant. Since the F_2 and BCP_1 (the inefficient parent) segregation patterns showed a goodness of fit consistent with a 3:1 and 1:1 ratio respectively, and the segregation of the backcross to Voyager showed all high zinc, one can conclude that zinc efficiency in this navy bean cross is controlled by a single gene, and that zinc efficiency is dominant over zinc inefficiency.

The midparent value of seed zinc concentration for 'Albion' and 'Voyager' was 25.6μg g^{-1}, and the mean value of the F_1 population was 28.4μg g^{-1} (Figure 11). According to a linear contrast, the F_1 value was significantly higher than that of the midparent ($p= 0.05$), which suggested that the gene controlling zinc efficiency shows

partial dominance. However, data derived from the F_2 and BC generations indicated that additive gene action was responsible for the majority of the variation for this trait (Table 10). The chi-square test for the adequacy of an additive/dominance model for zinc concentration indicated that the model was not disturbed by epistatic interactions (Table 9). Broad sense heritabilities for zinc, phytic acid phosphorus, non-phytic acid phosphorus and total phosphorus in seeds were 0.85, 0.17, 0.70, and 0.84, respectively (Table 11). The narrow sense heritability estimated for seed zinc concentration of 0.82 (Table 11) supports the conclusion that additive genetic variance had a major influence on trait expression. These high value broad and narrow sense heritability estimates for seed zinc concentration indicated the trait was not strongly influenced by environment, thus selection for increasing the level of seed zinc would be effective in early generations. A selection strategy that is appropriate for traits with high heritability estimates will be useful to develop inbred lines with increased levels of seed [Zn].

CONCLUSIONS

Based on the generation derived from 'Albion' x 'Voyager', breeding for higher seed [Zn] should be possible. Selections made at the highest levels of [Zn] indicated that seed [Zn] could be increased by nearly 50%. Such increases may improve the quality of the seed for human consumption.

Breeding for low PA-P in bean seed will be challenging because of the lack of variability for the trait combined with its low heritability. The use of targeted mutational breeding to inhibit a gene in the phytic acid synthesis pathway may be an approach to develop a low PA-P bean.

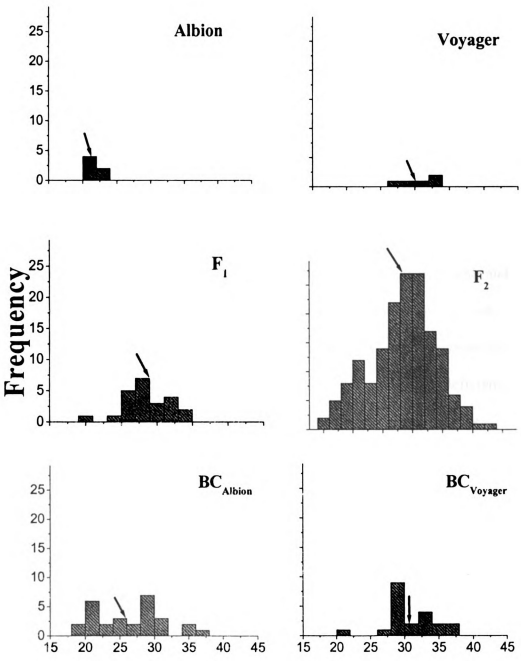

Figure 11. Frequency distribution of zinc concentration [Zn] in Albion and Voyager navy bean seed and four generations derived from the intermating of Albion and Voyager. The arrows indicate the mean [Zn] of each generation.

CHAPTER 3: SCREENING OF PHASEOLUS VULGARIS L. GENOTYPES FOR VARIABILITY IN PHYTIC ACID AND ZINC IN RAW AND THERMALLY PROCESSED SEED

INTRODUCTION

Phytic acid, the major form of phosphorus in cereal and legume seeds, reduces the bioavailability of zinc in humans (Turnlund et al., 1984). Marginal zinc deficiency is a widespread problem, especially for people consuming diets rich in cereals and legumes (Torre, 1991). Breeding for reduced levels of phytic acid along with increased zinc density in the seed may be a sustainable approach to increase the amount of bioavailable zinc in the diets of people suffering from zinc deficiency. The use of preparation procedures that reduce the amount of phytic acid by activating endogenous seed phytase may be another means to increase the percentage of bioavailable zinc in phytate rich meals.

The reports are conflicting on the effect of cooking and thermal processing (canning) bean seeds on the amount of phytic acid in bean seeds. Schlemmer et al. (1995) found that cooking beans in boiling water for two to three hours did not significantly reduce the phytic acid content of the seed. Greiner and Konietzny (1998) found cooking to reduce phytic acid levels 16 to 24% in bean. The same variability in results has been seen in thermal processing experiments with beans. Schlemmer et al. (1995) found canning had no effect, whereas Tabekhia and Luh (1980) showed a 70 to 75% reduction in phytic acid with canning. One reason for

the conflicting reports on how phytic acid is affected by thermal processing may be that there is genetic variability in bean seed for the conditions under which phytase is activated. Accordingly, this study was conducted to screen raw and thermally processed seed of 48 dry bean genotypes of diverse market classes for variability in levels of zinc, phytic acid, and total phosphorus. The objective of the study was to identify germplasm that may be valuable in a bean-breeding program to increase the density of zinc and reduce the amount of phosphorus in the phytic acid form in bean seeds.

MATERIALS AND METHODS

A nursery consisting of 48 dry bean genotypes representing several market classes of Middle American gene pool and one genotype of the Andean gene pool (Jacob's Cattle) was screened. The breeding lines and cultivars were planted at the Saginaw Valley Bean and Sugar Beet Research Farm near Saginaw, Michigan in 1999. Seed of the entries were precision-drilled with a tractor mounted air planter into four row plots in a Mistequay silty clay [fine-illitic (calcareous) frigid typic Haplaquolls] soil. Rows were 5 m long and spaced 0.5 m apart. Within row spacing was 0.12 m. The planting arrangement was a 7x7-balanced lattice with three replications. At planting a 21-7-0 and 4% Mn and 1% Zn fertilizer was applied in bands in each row at the rate of 224 kg ha^{-1}. A preplant herbicide application was made following recommendations by the Michigan Dry Bean Production Advisory Board Agronomist for 1999. Mature plants of the 48 entries were harvested from 6m of the middle two rows of each plot (6.1m^2) and threshed with an Almaco (Allen Machine Co., Nevada, IA) stationary plot thresher. The threshed seed was cleaned of

plant debris, broken and/or diseased grains, and soil particles and sized using 4 x 19mm slotted metal sieves. Plot yield and 100 seed weight was determined at 16% seed moisture. Seed samples were stored at 22°C and 75% relative humidity (Hosfield et al., 1999).

A 250g sample of seed from each entry was analyzed for seed moisture percentage using a moisture meter. Duplicate samples from each plot with a fresh-weight equivalent of 100g total solids (Hosfield and Uebersax, 1980) were placed in nylon mesh and equilibrated to 16% moisture in a humidified storage room. Remnant seed from the 250 g sample not placed in the nylon mesh bags was placed in small Kraft paper bags and stored at room temperature. The seed in the mesh bags was thermally processed according to the procedures of Hosfield and Uebersax (1980) and Hosfield and Uebersax et al. (1984 a,b). Thermal processing of the seed samples began by soaking at 21°C for 30 min and blanching at 88°C for 30 min. Soaking and blanching were done in distilled water adjusted to 0.025 mol L^{-1} calcium ion (Hosfield and Uebersax, 1980; Uebersax and Bedford, 1980). The soaking and blanching procedures produced well-hydrated beans with minimum bean damage, similar to beans soaked and blanched in the high-temperature systems common throughout the U.S. canning industry (Hosfield and Uebersax, 1980).

After bean samples were blanched, each was immersed in tap water (22°C) for 5 min to cool. Next samples were drained, weighed, and filled into number 303 (100 x 75 mm) tin cans and covered with boiling brine. The brine was prepared by adding 142.0g sucrose and 113.4g of NaCl to 9.1 kg of distilled water adjusted to 0.025 mol/L (100 ppm) calcium ion. The cans were exhausted at 90°C for 5 min in a water

filled exhaust box, sealed and cooked in a commercial retort without agitation for 45 min at 116°C and 10.4 x 10 Pa (15 Psi). After the beans were thermally processed, the cans were removed from the retort, cooled under running tap water (22°C) for 15 min, and stored inverted for a minimum of two weeks before cans were opened for evaluation.

The concentration of phosphorus, phytic acid, and zinc was measured on both the thermally processed and raw seed. Raw seed was the remnant from the 250 g initial sample from each entry. Prior to analyses, the raw seed was freeze dried in a Virtis, Genesis® freeze dryer. Seed was then ground to a fine powder with a coffee grinder. Thermally processed beans were blended into a paste in a blender, freeze-dried and ground to a fine powder in a Wiley mill with a 60-mesh screen. From this point the raw and thermally processed seed was handled similarly. For P and Zn analysis, 0.5 g of seed was weighed and placed in a ceramic crucible. The samples were ashed in a muffle furnace for 8 hours at 495°C. Samples were then acidified with 25ml 3N HNO_3. Samples were analyzed for Zn by atomic adsorption (Varian Spectra AA-20 plus) and for total P by the colorimetric method developed by Murphy and Riley (1963) with a Brinkman dipping probe with an 880 nm filter attached to the probe. Samples were analyzed for phytic acid (PA) by HPLC. Sample preparation for PA analysis in bean seed was conducted following the procedure of Lehrfeld (1980). PA was extracted from the samples by the addition of 3ml 0.5M HCl (trace element grade) to 0.100 g of sample. The acidified samples were mechanically agitated for 2 hours at 21°C. Samples were centrifuged at 12,000g for 15 minutes. The supernant was collected and diluted 1:5 (v/v) with

distilled, deionized water. The diluted sample (15 ml total) was passed through a Bond Elut strong anion exchange column (Varian, Walnut Creek, CA) for purification. The column was washed once with 10 ml 0.05 M HCl, and phytic acid was eluted with 3ml 2M HCl. The eluted fraction containing PA was air dried and dissolved in 5mM sodium acetate. The dissolved sample was filtered through a 2μm filter. PA levels in each sample were quantified by high performance liquid chromatography with a refractive index detector. The column used for analysis was a Waters Symmetry C18 column (3.9mm x 150mm) (Waters, Milford, MA) heated to a temperature of 40°C. Sodium acetate (5mM) was used as the solvent at a flow rate of 1.4 ml min^{-1}. Phytic acid dodecasodium salt from corn (*Z. mays* L.) (Sigma, St. Louis, MO) was used as a standard to determine PA concentration. The amount of P in the form of PA (PA-P) was determined by dividing the concentration of phytic acid by 3.55 (Lott at al., 2000)

Data was converted to concentration on a dry weight basis. Statistical analyses, including least significant differences among cultivars, were conducted using SAS (SAS Inst., Inc., 1997).

RESULTS AND DISCUSSION

The identification, commercial class designation, seed coat color, seed weight, and yield of the 48 dry bean genotypes screened for seed Zn, P, and PA-P are presented in Table 12. In the raw seed, PA-P values ranged from 1.11 mg g^{-1} to 2.14 mg g^{-1} with a mean of 1.65 mg g^{-1}(Table 13). The cultivar with the lowest seed PA-P (Carioca) was not significantly different from the mean PA-P value of all the cultivars. PA-P made up 30 to 50% of the total seed phosphorus. The PA-P values

Table 12. The commercial class, seed coat color, seed weight and yield of 48 dry bean genotypes screened for the concentration of phytic acid phosphorus, total phosphorus, and zinc in the seed.

Identification	Commercial class	Seed coat color	†Seed weight (g ·100 seed $^{-1}$)	†Yield (kg·ha^{-1})
BlackTurtle Soup	Tropical black	Black	23.4	2498
800242	Small white	White	20.2	2687
Seafarer	Navy	White	21.2	3012
Domino	Tropical black	Black	22.5	2628
Sanilac	Navy	White	18.6	2864
Tuscola	Navy	White	20.3	2408
8217-III-24	Navy	White	21.0	2427
Jalpataqua	Tropical black	Black	25.6	2602
Nep-2	Small white	White	20.1	2944
San Fernando	Tropical black	Black	20.4	2539
Bunsi	Navy	White	21.2	2637
ICA Pijao	Tropical black	Black	22.2	2861
Aurora	Small white	White	17.2	2894
P766	Undefined	Black	21.9	2275
Jamapa	Tropical black	Black	22.7	3190
Protop-Pl	Pinto	Brown mottle	29.7	2143
FF4-13-MMMM	Tropical black	Black	23.0	2743
C-20	Navy	White	20.1	2715
Fleetwood	Navy	White	19.8	3142
Mexico 12-1	Undefined	Brown	21.6	1915
Carioca	Pinto	Brown mottle	26.2	1347
Laker	Navy	White	19.6	2485
Swan Valley	Navy	White	19.5	2818
Midnight	Tropical black	Black	20.6	2235
BAT 41	Small red	Red	19.8	1950
15-R-148	Small red	Red (shiny)	20.4	1950
BAT 1507	Small red	Red (shiny)	24.6	2734
BAT 1376	Small red	Red	24.4	1937
BAC 95	Small red	Red (shiny)	24.0	2950
Harblack(opaque)	Tropical black	Black	20.0	2577
Harblack(shiny)	Tropical black	Black(shiny)	21.0	2763
Cumulus	Navy	White	22.9	2830
N84004	Navy	White	20.2	2984
C-20 Mutant	Navy	White	20.4	2452
Jacob's Cattle	Heirloom	Red/white	50.8	1330
Huron	Navy	White	22.0	1554
Mayflower	Navy	White	21.7	2915
Albion	Navy	White	19.4	2124
Newport	Navy	White	21.3	2899
N81099	Navy	White	21.5	3344

Table 12 (cont'd)

Identification	Commercial class	Seed coat color	†Seed weight (g ·100 seed $^{-1}$)	†Yield (kg·ha^{-1})
Huetar	Small red	Red	23.7	2151
Black Jack	Tropical black	Black	23.0	2572
Raven	Tropical black	Black	19.5	2336
T-39	Tropical black	Black	22.1	2644
Shiny Crow	Tropical black	Black	26.0	3039
BunsixRaven	Tropical black	Black	20.7	2654
Garnet	Small red	Red	29.7	2035
Rufus	Small red	Red	35.4	3024
% CV			7.2	15.9
LSD (0.05)			2.6	669

† Seed weight and yield are based on seed at 16% moisture. Plants were grown at the Michigan State University Bean & Beet Research Farm in 1999 (Hosfield et al., 1999).

Table 13. The concentration of zinc [Zn], [P], and the phytic acid form of phosphorus [PA-P] in raw and thermally processed seed of 48 bean genotypes.

Identification	Seed zinc ($\mu g \cdot g^{-1}$)		Seed phosphorus ($mg \cdot g^{-1}$)		Seed phytic acid phosphorus ($mg \cdot g^{-1}$)	
	Raw	Processed	Raw	Processed	Raw	Processed
Black Turtle Soup	30.2	25.1	4.08	3.71	1.31	0.89
800242	30.2	23.9	4.28	4.16	2.11	1.33
Seafarer	30.1	21.1	4.83	3.58	1.71	1.28
Domino	27.1	28.0	4.29	4.25	1.55	1.15
Sanilac	26.4	23.7	4.74	4.29	1.97	1.46
Tuscola	43.3	26.1	4.51	4.21	1.86	1.67
8217-III-24	32.4	26.8	4.77	4.04	1.71	1.09
Jalpataqua	29.5	26.7	4.44	3.90	1.79	1.37
Nep-2	29.2	25.5	4.48	4.26	2.00	1.24
San Fernando	31.4	31.1	4.81	4.16	1.97	1.09
Bunsi	27.7	26.6	4.69	3.99	2.14	1.49
ICA Pijao	27.1	25.2	4.05	4.04	2.00	0.98
Aurora	29.5	24.7	4.45	3.90	1.83	1.23
P766	29.2	34.5	4.67	4.41	2.14	1.55
Jampa	34.0	25.6	4.02	3.65	1.62	1.07
Protop-P1	33.9	26.9	5.26	4.34	1.77	1.19
FF4-13-MMMM	29.0	25.1	4.15	3.84	1.63	1.08
C-20	66.9	22.4	4.41	3.84	1.79	1.04
Fleetwood	39.7	20.7	4.47	3.86	1.34	1.21
Mexico 12-1	27.9	25.5	3.92	3.58	1.54	0.86
Carioca	31.4	21.9	3.92	3.29	1.11	0.94
Laker	26.5	26.7	4.82	4.32	1.71	1.22
Swan Valley	25.1	21.3	4.11	3.81	1.49	1.19
Midnight	43.3	26.5	4.56	4.04	1.82	1.21
BAT 41	29.1	25.7	5.89	4.42	1.93	1.42
15-R-148	32.3	27.0	4.08	4.05	1.5	0.83
BAT 1507	24.6	29.1	4.02	3.86	1.34	1.11
BAT 1376	29.8	22.9	4.47	4.46	1.51	1.36
BAC 95	26.0	24.4	3.99	3.59	1.36	0.79
Harblack (opaque)	40.0	23.8	4.60	4.16	1.58	0.90
Harblack (shiny)	27.5	28.0	4.46	3.89	1.48	1.20
Cumulus	28.0	22.3	3.97	3.88	1.42	0.96
N84004	36.2	24.7	4.25	3.94	1.76	1.47
C-20 Mutant	27.1	23.5	4.28	3.77	1.68	1.11
Jacob's Cattle	46.2	23.0	4.08	3.81	1.43	1.09
Huron	26.6	22.9	4.31	4.10	1.43	1.14
Mayflower	30.9	24.1	4.22	4.12	1.55	1.31
Albion	29.9	23.9	4.42	4.28	1.68	1.26
Newport	26.5	20.5	3.68	3.63	1.57	1.11

Table 13 (cont'd)

Identification	Seed zinc ($\mu g \cdot g^{-1}$)		Seed phosphorus ($mg \cdot g^{-1}$)		Seed phytic acid phosphorus ($mg \cdot g^{-1}$)	
	Raw	Processed	Raw	Processed	Raw	Processed
N81099	39.4	23.0	4.22	4.32	1.69	1.57
Huetar	33.7	26.2	4.10	3.91	1.33	0.98
Black Jack	26.8	27.0	4.39	4.34	1.55	1.50
Raven	26.3	23.8	4.77	3.83	1.75	0.86
T-39	40.6	32.4	4.33	4.16	1.56	0.97
Shiny Crow	24.3	24.2	3.95	3.97	1.53	0.89
Bunsi x Raven	33.1	26.7	4.49	3.97	1.66	1.08
Garnet	25.9	22.9	4.00	3.85	1.57	1.06
Rufus	27.2	21.3	4.16	3.89	1.61	1.12
mean	31.1	25.1	4.37	3.99	1.65	1.17
SE	0.87	0.37	0.06	0.03	0.04	0.03
LSD (0.05)	12.1	5.1	0.78	0.42	0.54	0.41

for the raw seed were lower than the 60 to 80% that have been reported in the literature (Reddy et al., 1989). One reason for the reduced levels of PA-P in this study may be that the bean seed was harvested in October of 1999 but not screened until 2002. Beans were stored where summertime temperatures may have reached 25° C. At room temperature storage for 1.5 years, white beans were shown to lose 10% of the PA (Ockenden et al., 1997). Therefore, one might expect some breakdown of PA at storage temperatures above RT and especially if they were stored for more than one year. The thermally processed seed had on average 1.17 mg g-1 PA-P, and a range of 0.8 to 1.67 mg g-1 PA. These data did not indicate significant genetic variability for PA-P of thermally processed seed. There was on average 29% reduction PA-P than the raw seed (Figure A3 and Table 13). A study by Greiner and Konietzny (1998) indicated a similar reduction in seed phytic acid following a 15 h soak in water and cooking in boiling water for 2 hours. They found endogenous phytases to be responsible for the reduction in phytic acid. Such reductions in PA-P many be beneficial to human nutrition. According to nutrition studies where humans were fed diets consisting of low phytic acid maize (25% of total seed P is PA-P) and normal maize (75% of total seed P is PA-P), the zinc absorbance of the low PA maize was 30% while the absorbance was only 17% for the normal maize (Adams, 2000).

The genetic variability in raw seed [P] ranged from 3.7 mg g^{-1} to 5.9 mg g^{-1} with a mean of 4.4 mg g^{-1} (Figure A4 and Table 13). Thermally processed seed [P] ranged from 3.3 mg g^{-1} to 4.5 mg g^{-1} with a mean of 4.0 mg g^{-1}. Thermally processed seed had on average 8% less seed P than raw seed. This phosphorus either leached into

the soak water or the brine, but since these components of the thermally processed beans were not fractionated, the fate of phosphorus in thermally processing samples is unknown.

The zinc concentration of raw seed ranged from 24.3μg g^{-1} to 46.2μg g^{-1} with a mean of 31.1-μg g^{-1}. The zinc concentration of the thermally processed seed had much less variability as compared with the raw seed (Figure A5). The concentrations ranged from 20.5μg g^{-1} to 34.5μg g^{-1} with a mean of 25.1 μg g^{-1}. The thermally processed seed had, on average, 17% less zinc than the raw seed (Table 13). There was considerable variability in the amount of Zn that leached from the seed during thermal processing. Fleetwood lost 48% of its seed Zn during processing, while Shiny Crow, Black Jack, Laker, and San Fernando lost less than 1% of this mineral during processing (Table 13). Studies have shown a loss of nutrients in the effluent from bean processing (Meiners et al., 1976 and Muggio et al., 1985). Rodriguez-Burger et al. (1998) found limited reduction in seed Zn in black beans following cooking, but they only investigated one cultivar. The large cultivar differences for the amount of Zn lost during processing may be related to the location in the seed that Zn is stored. Based on studies with two black bean cultivars, Moraghan and Grafton (2002) found 39% to 44% of the total seed zinc was located in the seed coat. Although no literature could be found on the leaching of zinc from different parts of the seed, one would expect the zinc in the seed coat to leach more rapidly from the seed than zinc in the cotyledons and embryo. Despite the reduction in seed zinc following thermal processing, there existed significant variation for [Zn] among the cultivars. The genotype P766, a black seeded bean, had 27% more seed [Zn] after

after processing than the average of the genotypes. Additional screening of P766 is necessary to determine its value in a breeding program for improving the zinc status of dry bean. Breeding for increased seed [Zn] may be a promising way to increase the bioavailability of zinc for human consumption. According to experiments with rat models, the % of Zn bioavailability tends to remain constant independent of the amount of zinc in the seed (Ruel and Bouis, 1998). Therefore, Zn enhanced seed should have a net increase in bioavailable Zn.

CONCLUSIONS

Screening of the 48 dry bean genotypes for seed Zn, PA-P, and total P, indicated that substantial variability existed among genotypes for Zn in raw seed but there was less variability in the thermally processed seed as much of the Zn was lost during the processing. The variability for PA-P and total P among the 48 genotypes was minimal. There was not significant variability in the PA-P of raw or thermally processed seed, but the level of PA-P was reduced 29% on average by thermal processing.

APPENDIX

Table A1. Analysis of variance for phosphorus in the phytic acid form [PA-P], non phytic acid P [Non PA-P], and total phosphorus concentration in the seed of Voyager and Albion navy bean cultivars grown under three levels of phosphorus and five levels of zinc †.

Trait	Source of variation	Degrees of freedom	Mean square	F statistic	Pr > F
PA-P	Replication	3	0.07	1.7	0.18
	Cultivar	1	0.01	0.22	0.64
	P	2	33	742	< .0001
	Zn	4	1.80	40	< .0001
	P * Zn	8	0.62	14	< .0001
	Cultivar * P	2	0.03	0.64	0.53
	Cultivar * Zn	4	0.05	1.04	0.39
	Cultivar * P * Zn	8	0.04	0.88	0.53

Trait	Source of variation	Degrees of freedom	Mean square	F statistic	Pr > F
Non PA-P	Replication	3	0.04	1.0	0.38
	Cultivar	1	7.24	209	<.0001
	P	2	7.3	210	< .0001
	Zn	4	0.83	24	< .0001
	P * Zn	8	0.16	4.6	0.0001
	Cultivar * P	2	1.08	31	< .0001
	Cultivar * Zn	4	0.50	14.	<.0001
	Cultivar * P * Zn	8	0.10	2.8	0.01

Trait	Source of variation	Degrees of freedom	Mean square	F statistic	Pr > F
Total P	Replication	3	0.06	1.6	0.20
	Cultivar	1	6.8	176	<.0001
	P	2	71	1848	< .0001
	Zn	4	5.1	132	< .0001
	P * Zn	8	1.3	33	< .0001
	Cultivar * P	2	0.77	20	< .0001
	Cultivar * Zn	4	0.43	11	< .0001
	Cultivar * P * Zn	8	0.17	4.4	0.0002

† Voyager and Albion are zinc efficient and zinc inefficient cultivars respectively.

Table A2. Analysis of variance for content per experimental unit (seed of 3 plants) of phosphorus in the phytic acid form (PA-P), non phytic acid P (non PA-P), and total phosphorus in Voyager and Albion navy bean cultivars grown under three levels of phosphorus and five levels of zinc †.

Trait	Source of variation	Degrees of freedom	Mean square	F statistic	Pr > F
PA-P	Replication	3	173	5.3	0.0021
Content	Cultivar	1	4762	146	<.0001
	P	2	3074	945	<.0001
	Zn	4	358	11	<.0001
	P * Zn	8	357	11	<.0001
	Cultivar * P	2	1320	41	<.0001
	Cultivar * Zn	4	1159	36	<.0001
	Cultivar * P * Zn	8	266	8	<.0001

Trait	Source of variation	Degrees of freedom	Mean square	F statistic	Pr > F
Non	Replication	3	257	11	<.0001
PA-P	Cultivar	1	37	1.6	0.22
Content	P	2	1000	419	<.0001
	Zn	4	470	20	<.0001
	P * Zn	8	242	10	<.0001
	Cultivar * P	2	465	19	<.0001
	Cultivar * Zn	4	305	13	<.0001
	Cultivar * P * Zn	8	153	6.4	<.0001

Trait	Source of variation	Degrees of freedom	Mean square	F statistic	Pr > F
Total	Replication	3	821	13	<.0001
P	Cultivar	1	3959	64	<.0001
Content	P	2	7549	1218	<.0001
	Zn	4	1619	26	<.0001
	P * Zn	8	1077	17	<.0001
	Cultivar * P	2	2341	38	<.0001
	Cultivar * Zn	4	1049	42	<.0001
	Cultivar * P * Zn	8	6211	13	<.0001

† Voyager and Albion are zinc efficient and zinc inefficient cultivars respectively.

Table A3. Analysis of variance for seed yield, seed zinc concentration [Zn], seed zinc per experimental unit, and percent phosphorus in the phytic acid form for Voyager and Albion navy bean cultivars grown under three levels of phosphorus and five levels of zinc. †

Trait	Source of variation	Degrees of freedom	Mean square	F statistic	Pr > F
Seed Yield	Replication	3	52	14	<.0001
	Cultivar	1	477	130	<.0001
	P	2	961	262	<.0001
	Zn	4	207	56	<.0001
	P * Zn	8	89	24	<.0001
	Cultivar * P	2	68	18	<.0001
	Cultivar * Zn	4	96	26	<.0001
	Cultivar * P * Zn	8	21	5.8	<.0001

Trait	Source of variation	Degrees of freedom	Mean square	F statistic	Pr > F
Seed [Zn]	Replication	3	6.4	3.2	0.03
	Cultivar	1	476	238	<.0001
	P	2	2704	1353	<.0001
	Zn	4	405	202	<.0001
	P * Zn	8	30	15	<.0001
	Cultivar * P	2	45	23	<.0001
	Cultivar * Zn	4	25	12	<.0001
	Cultivar * P * Zn	8	16	7.9	<.0001

Trait	Source of variation	Degrees of freedom	Mean square	F statistic	Pr > F
Seed Zinc Content	Replication	3	4829	14	<.0001
	Cultivar	1	7491	218	<.0001
	P	2	8112	24	<.0001
	Zn	4	3891	113	<.0001
	P * Zn	8	2526	7.4	<.0001
	Cultivar * P	2	1416	0.41	0.66
	Cultivar * Zn	4	7263	2.1	0.09
	Cultivar * P * Zn	8	7356	2.1	0.04

Trait	Source of variation	Degrees of freedom	Mean square	F statistic	Pr > F
%	Replication	3	25	1.2	0.30
P as PA	Cultivar	1	974	48	<.0001
	P	2	995	49	< .0001
	Zn	4	3.5	0.17	0.95
	P * Zn	8	31	1.5	0.16
	Cultivar * P	2	162	8.0	0.0006
	Cultivar * Zn	4	64	3.2	0.02
	Cultivar * P * Zn	8	9.7	0.48	0.86

† Voyager and Albion are zinc efficient and zinc inefficient cultivars respectively.

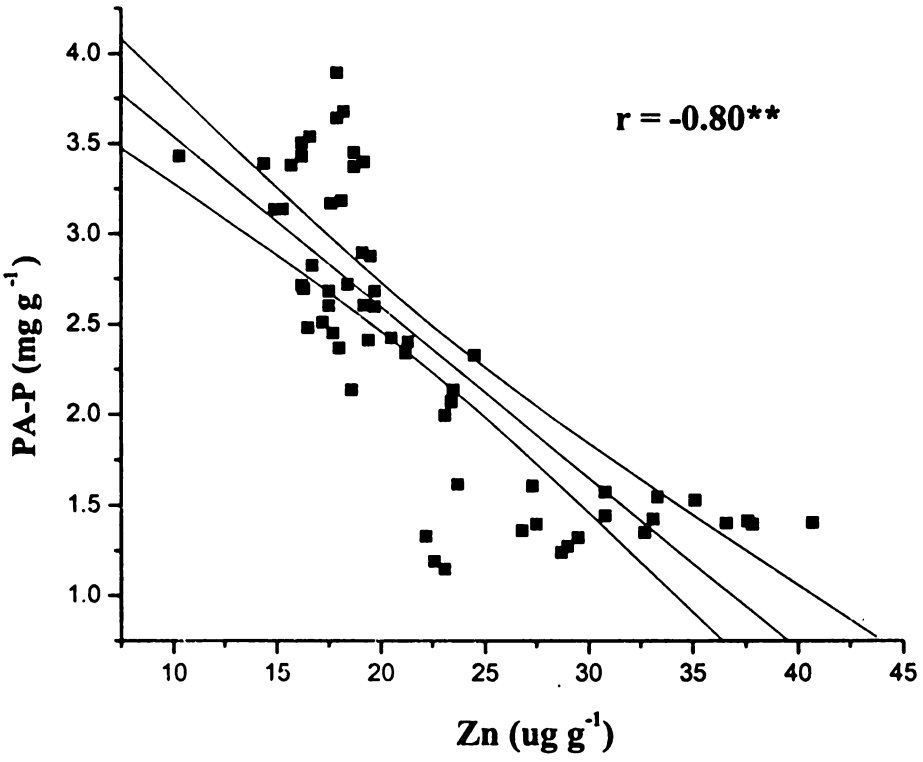

Figure A1. Correlations between seed zinc concentration [Zn] and the concentration of phosphorus in the phytic acid form [PA-P] of Albion navy beans grown under three levels of phosphorus and five levels of zinc.

** significant at P $\leq$0.05

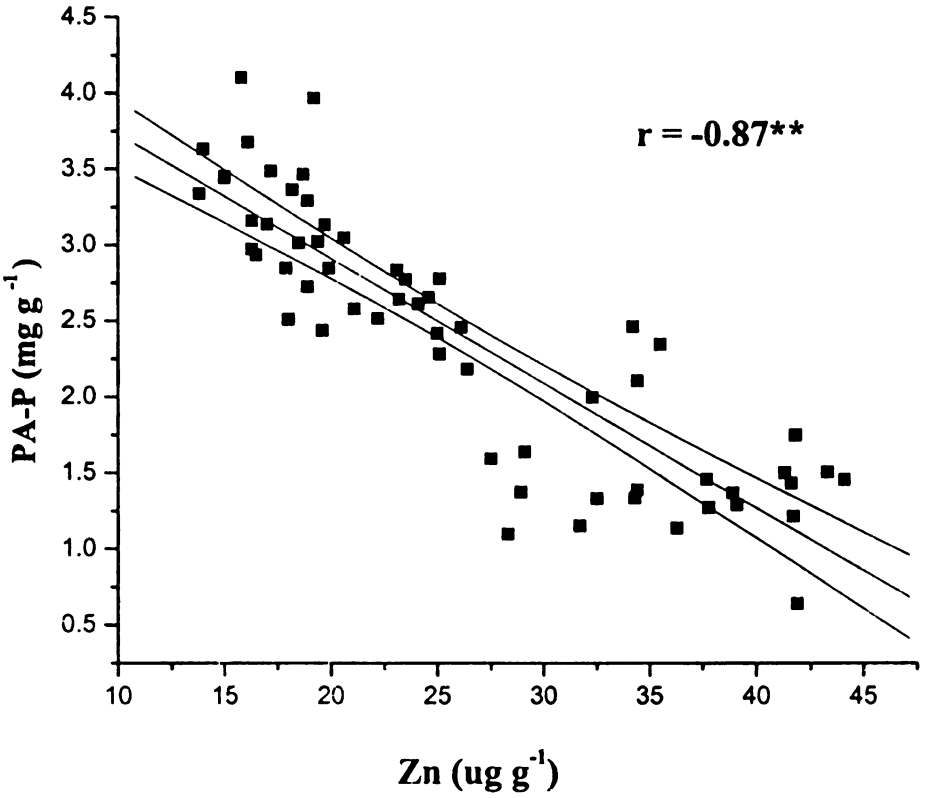

Figure A2. Correlations between seed zinc concentration [Zn] and the concentration of phosphorus in the phytic acid form [PA-P] of Voyager navy beans grown under three levels of phosphorus and five levels of zinc.

** significant at P ≤0.05

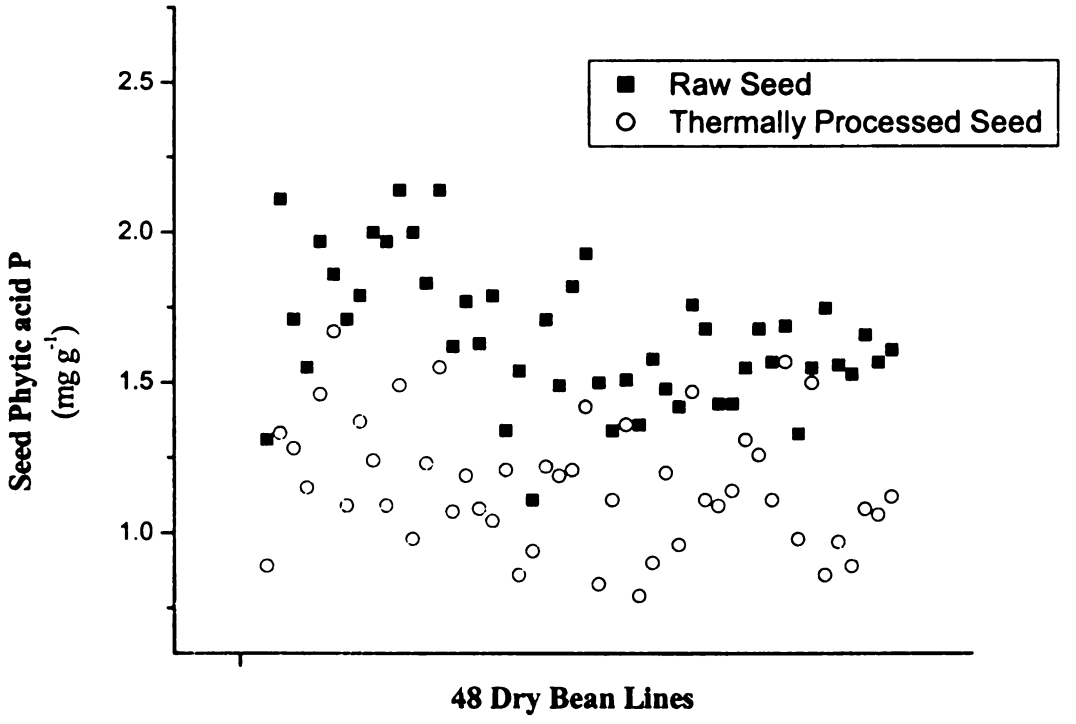

Figure A3. Phytic acid phosphorus concentration [PA-P] of raw and thermally processed seed of 48 diverse genetic stocks of dry bean grown in a nursery in 1999.

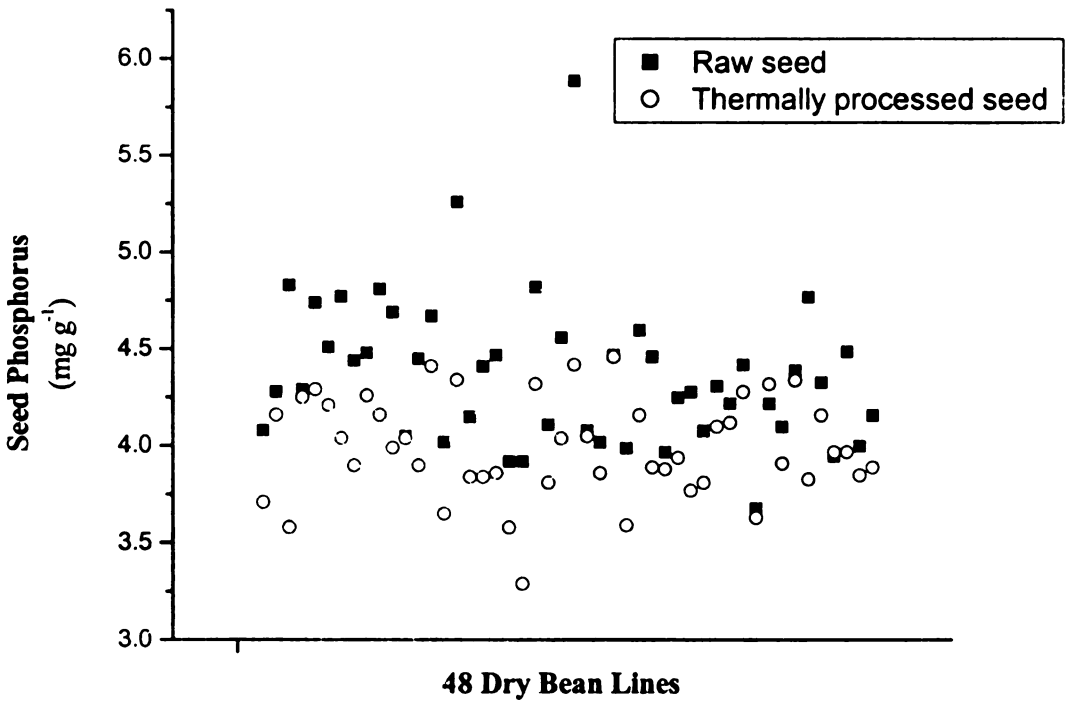

Figure A4. Phosphorus concentration [P] of raw and thermally processed seed of 48 diverse genetic stocks of dry bean grown in a nursery in 1999.

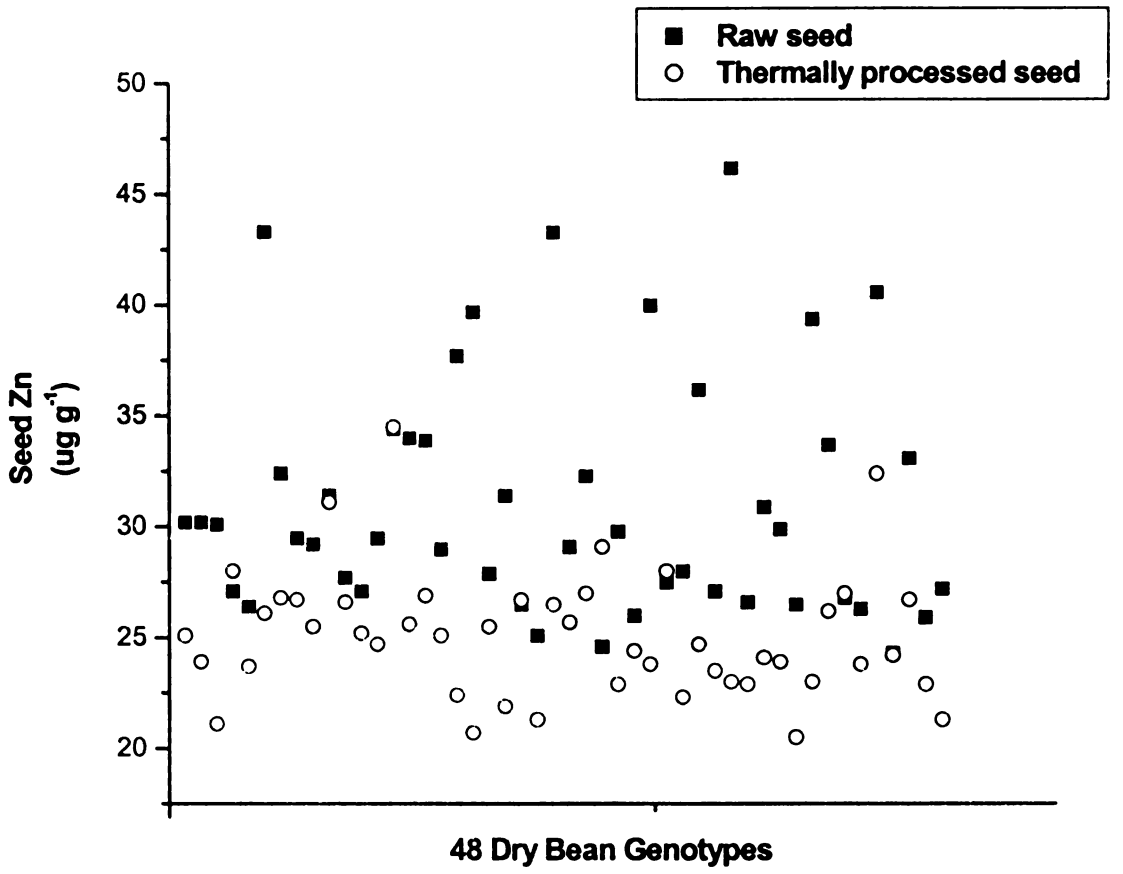

Figure A5. Zinc concentration [Zn] of raw and thermally processed seed of 48 diverse genetic stocks of dry bean grown in a nursery in 1999.

LITERATURE CITED

Abu-Abied, M., Holland, D. 1994. The gene c-ino1 from Citrus paradisi is highly homologous to tur1 and ino1 from yeast and Spiroleda encoding for myo-inositol phosphate synthase. Plant Physiol. 106:1689.

Adams, C., V. Raboy, N. Krebs, J. Westcott, S. Lei, and M. Hambidge. 2000. The effect of low-phytic acid corn mutants on zinc absorption. FASEB J 14:A510-A510.

Allard, R.W. 1960. Principles of Plant Breeding Wiley, New York.

Ambler, J. E., Brown, J.C. 1969. Cause of Differential Susceptibility to Zinc Deficiency in Two Varieties of Navy Bean (Phaseolus vulgaris L.). Agronomy Journal 61: 41-43.

Asada, K.K., K. Tanaka, Z. Kasai. 1969. Formation of phytic acid in cereal grains. Ann NY Acad Sci 165:801-814.

Barampama, Z., and R.E. Simard. 1993. Nutrient Composition, Protein-Quality and Antinutritional Factors of Some Varieties of Dry Beans (Phaseolus-vulgaris) Grown in Burundi. Food Chemistry 47:159-167.

Barrientos, L., Scott, J.J., Murthy, P.P.N (1994). Specificity of Hydrolysis of Phytic Acid by Alkaline Phytase from Lily Pollen. Plant Physiol. 106: 1489-1495.

Bliss, F.A., and B. Quebedeaux 1988. Horticulture and human health : contributions of fruits and vegetables: proceedings of the 1st International Symposium on Horticulture and Human Health Prentice Hall, New Jersey

Bliss, F.A. 1999. Nutritional improvement of horticultural crops through plant

breeding. Hortscience 34:1163-1167.

Bouis, H.E. 1999. Economics of enhanced micronutrient density in food staples. Field Crops Research 60:165-173.

Brearley, C.A., Hanke, D.E. 1996. Metabolic evidince for the order of addition of individual phosphate esters to the myo-inositol moiety of inositol hexakisphosphate in the duckweed Spirodela polyrhiza L. Biochem. J. 314:227-233.

Brearley, C.A., Hanke, D.E. 1996. Inositol phosphates in the duckweed Spirodela polyrhiza L. Biochem. J. 3114:215-225.

Brown K.H., W.S.E., Peerson, J.M. 2001. The importance of zinc in human nutrition and estimation of the global prevalance of zinc deficiency. Food and Nutrition Bulletin 22:113-125.

Brown, A. L., Krantz, B.A., Eddings, J.L. (1970). "Zinc-Phosphorus Interactions as Measured by Plant Response and Soil Analysis." Soil Science 110(6): 415-420.

Chang, R. 1977. Phytate: Removal From Whole Dry Beans By Enzymatic Hydrolysis and Diffusion. J Food Sci. 42:1098-1101.

Cheryan, M. 1980. Phytic Acid Interactions in Food Systems. Crit. Rev. Food Sci.Nutr. 13:297-335.

Cosgrove, D.J. 1980. Inositol Phosphates, Their Chemistry, Biochemistry and Physiology Elsevier, Amsterdam.

Ertl, D.S., Young, K.A., Raboy, V. 1998. Plant Genetic Approaches to

Phosphorus Management in Agricultural Production. J. Environ. Qual. 27:299-304.

Foolad, M.R. 1996. Genetic analysis of salt tolerance during vegetative growth in tomato, Lycopersicon esculentum Mill. Plant Breeding 115:245-250.

Frossard, E., M. Bucher, F. Machler, A. Mozafar, and R. Hurrell. 2000. Potential for increasing the content and bioavailability of Fe, Zn and Ca in plants for human nutrition. Journal of the Science of Food and Agriculture 80:861-879.

Gamble, E.E. 1962. Gene effects in corn (Zea mays L.): I Separation and relative importance of gene effects for yield. Canadian J. Plant Sci. 42:339-348.

Gibson, D. M., Ullah, A.B.J. (1990). Phytases and Their Action on Phytic Acid. Inositol Metabolism in Plants. D. J. Morre, Boss, W.F., Loewus, F.A. Chicago, Wiley-Liss.

Greenwood, J.S., Bewley, J.D. 1984. Subcellular distribution of phytin in the endosperm of developiong castor bean: a possibility for its synthesis in the cytoplasm prior to deposition within protein bodies. Planta 160:113-120.

Greiner, R., Alminger, M.L. (2001). "Stereospecificity of Myo-Inositol Hexakisphosphate Dephospphorylation by Phytate-Degrading Enzymes of Cereals." J. Food Biochem. 25: 229-248.

Greiner, R., Konietzny, U. 1998. Endogenous Phytate-Degrading Enzymes Are Responsible For Phytate Reduction While Preparing Beans (*Phaseolus vulgaris*). J Food Proces. Preserv. 29:321-331.

Hambidge, M. 2000. Human Zinc Deficiency. J. Nutr. 130.

Hara, K., Yagi, M., Koizumi, N., Kusano, T., Sano, H. 2000. Screening of wound-responsive genes identifies an immediate-early expressed gene encoding a highly charged protein in mechanically wounded tobacco plants. Plant Cell Physiol. 41:684-691.

Hegeman, C.E., Good, L.L., Grabau, E.A. 2001. Expression of D-myo-Inositol-3-Phosphate Synthase in Soybean. Implications for Phytic Acid Biosynthesis. Plant Physiol. 125:1941-1948.

Holthaus, J.F., J.B. Holland, P.J. White, and K.J. Frey. 1996. Inheritance of beta-glucan content of oat grain. Crop Science 36:567-572.

Hosfield, G.L., Bushey, S.M., Marchetti, B.L., Engleright, R., Kelly, J.D., Taylor, J. 1999. USDA, Agricultural Research Service Dry Bean Breeding Program Results For 1999. Michigan State University Agricultural Experiment Station, Saginaw Valley.

Hosfield, G.L., A. Ghaderi, and M.A. Uebersax. 1984a. A Factor-Analysis of Yield and Sensory and Physicochemical Data from Tests Used to Measure Culinary Quality in Dry Edible Beans. Canadian Journal of Plant Science 64:285-293.

Hosfield, G.L., M.A. Uebersax, and T.G. Isleib. 1984b. Seasonal and Genotypic Effects on Yield and Physicochemical Seed Characteristics Related to Food Quality in Dry, Edible Beans. Journal of the American Society for Horticultural Science 109:182-189.

Hosfield, G.L., and M.A. Uebersax. 1980. Variability in Physicochemical

Properties and Nutritional Components of Tropical and Domestic Dry Bean Germplasm. Journal of the American Society for Horticultural Science 105:246-252.

House, W.A., Welch, R.M., Van Campen, D.R. 1982. Effect of Phytic acid on the Absorption, Distribution, and Endogenous Excretion of Zinc in Rats. J. Nutr. 112: 941.

Hunt, J.R., Matthys, L.A., Johnson, L.K. 1998. Zinc absorption, mineral balance, and blood lipids in women consuming controlled lactoovegetarian and omnivorous diets for 8 weeks. Am. J. Clin. Nutr. 67: 421.

International Zinc Association 2000. Zinc and Human Health. International Zinc Association. Brussels, Belgium.

Ishitani, M., Majumder, A.L., Bornhouser, A., Michalowski, C.B., Jensen, R.G., Bohnert, H.J. 1996. Coordinate transcriptional induction of myo-inositol metabolism during environmental stress. Plant J. 9.

Jaffe, W.G. 1973. Nutritional aspects of common beans and other legume seeds as animal and human foods. USAID, Brazil.

Jarrell, W. M., Beverly, R.B. (1981). "The Dilution Effect in Plant Nutrition Studies." Advances in Agronomy 34: 197-224.

Johnson, M.D. 1994. The Arabidopsis thaliana myo-inositol 1-phosphate synthase (EC 5.5.1.4). Plant Physiol. 105:1023-1024.

Judy, W., J. Melon, G. Lessman, B. Ellis, and J. Davis. 1965. Zinc fertilization of pea beans, corn and sugarbeets in 1964. Mich. Agric. Exp. Stn. Res. Rep. 33., East Lansing, MI.

Kannan, S., Nielsen, S., Rodriguez-Burger, A., Mason, A.C. 2001. Iron and Zinc Bioavailability in Rats Fed Intrinsically Labeled Bean and Bean-Rice Infant Weaning Food Products. J. Agr. Food Chem. 49:5063-5069.

Keller, R., Brearley, C.A., Trethewey, R.N., Muller-Rober, B. 1998. Reduced inositol content and altered morphology in transgenic potato plants inhibited for 1D-myo-inositol-3-phosphate synthase. The Plant Journal 16: 403-410.

Kelly, J.D. 2000. Remaking Bean Plant Architecture for Efficient Production. Adv. Agr. 71: 109-143

Kikunaga, S., Katoh, Y., Takahashi, M. (1991). Biochemical Changes in Phosphorus Compounds and in the Activity of Phytase and alpha-Amylase in the Rice (Oryza sativa) Grain during Germination. J. Sci. Food Agric. 56: 335-343.

Knuckles, B.E., Kuzmicky, D.D., Gumbmann, M.R., Betschart, A.A. 1989. Effect of Myo-Inositol Phosphate Esters on In Vitro and In Vivo Digestion of Protein. J. Food Sci. 54:1348-1350.

Larson, S.R., Young, K.E., Cook, A., Blake, T.K., Raboy, V. 1998. Linkage mapping of two mutations that reduce phytic acid content of barley grain. Theor. Appl. Genet. 98:141-146.

Larson, S.R., Raboy, V. 1999. Linkage mapping of maize and barley myo-inositol 1-phosphate synthase DNA sequences: correspondence with a low phytic acid mutation. Theor. Appl. Genet 99:27-36.

Larson, S.R., J.N. Rutger, K.A. Young, and V. Raboy. 2000. Isolation and genetic

mapping of a non-lethal rice (Oryza sativa L.) low phytic acid 1 mutation. Crop Science 40:1397-1405.

Lemtiri-Chlieh, F., MacRobbie, E.A.C., Brearley, C.A. 2000. Inositol hexakisphosphate is a physiological signal regulating the K+-inward rectifying conductance in guard cells. Proc. Natl. Acad. Sci. 97:8687-8692.

Loewus, F.A., Everard, J.D., Young, K.A.1990. Inositol Metabolism: Precursor Role and Breakdown. In: Inositol Metabolism in Plants Wiley-Liss: New York.

Loewus, F.A., Murthy, P.N. 2000. myo-Inositol metabolism in plants. Plant Science 150:1-19.

Lolas, G.M., N, Palmidis, P. Markakis 1976. The phytic acid-total phosphorus relationship in barley, oats, soybeans, and wheat. Cereal Chem. 53:867-871.

Lolas, G.M., Markakis, P. 1977. The Phytase of Navy Beans (Phaseolus vulgaris). J. Food Sci. 42:1094-1098.

Loneragan, J. F., Grove, T.S., Robson, T.D., Snowball, K. (1979). "Phosphorus Toxicity as a Factor in Zinc-Phosphorus Interactions in Plants." Soil Sci. Soc. Am. J. 43: 966-972.

Loneragan, J. F., Grunes, D.L., Welch, R.M., Aduayi, E.A., Tengah, A., Lazar, V.A., Cary, E.E. (1982). "Phosphorus Accumulation and Toxicity in Leaves in Relation to Zinc Supply." Soil Sci. Soc. Am. J. 46: 345-352.

Lonnerdal, B., Sandberg, A., Sandstrom, B., Kunz, C. 1989. Inhibitory Effects of

Phytic Acid and Other Inositol Phosphates on Zinc and Calcium Absorption in Suckling Rats. J. Nutr. 119: 211

Lott, J.N.A., Buttrose, M.S. 1977. Globoids in Protein Bodies of Legume Seed Cotyledons. Aust. J. Plant Physiol. 5:89-111.

Lott, J.N.A., Greenwood, J.S., Batten, G.D. 1995. Mechanisms and Regulation of Mineral Nutrient Storage During Seed Development, In J. Kiegl, Galili, G., ed. Seed Development and Germination. Marcel Dekker, Inc., New York.

Lott, J.N.A., Ockenden, I., Raboy, V., Batten, G.D. 2000. Phytic acid and phosphorus in crop seeds and fruits: a global estimate. Seed Science Research 10:11-33.

Lynch, J., Lauchli, A., Epstein, E. 1991. "Vegetative Growth of the Common Bean in Response to Phosphorus Nutrition." Crop Sci. 31: 380-387.

Marschner, H., Ed. 1997. Mineral Nutrition of Higher Plants. New York, Academic Press.

Mather, K. and Jinks, J.L. 1971. Introduction to Biometrical Genetics Cornell University Press, Ithaca, NY.

McKenzie, D. B., Soper, R.J. 1983. "Zinc Regulation in Black Beans (Phaseolus vulgaris L.)." Commun. In Soil Sci. Plant Anal. 14(8): 709-725.

Meiners, C.R., N.L. Derise, H.C. Lau, S.J. Ritchey, E.W. Murphy 1976. Proximate composition and yield of raw and cooked mature dry legumes [Beans, peas, lentils]. J.Agric. Food Chem. 24 (6): 1122-1126.

Miller, G.A., V.L. Youngs, and E.S. Oplinger. 1980. Effect of Available Soil-

Phosphorus and Environment on the Phytic Acid Concentration in Oats. Cereal Chemistry 57:192-194.

Moraghan, J. T. (1980). "Effect of Soil Temperature on Response of Flax to Phosphorus and Zinc Fertilizers." Soil Science 129(5): 290-296.

Moraghan, J. T., Mascagni, H.J. (1991). Environmental and Soil Factors Affecting Micronutrient Deficiencies and Toxicities. Micronutrients in Agriculture. Madison, SSSA. 4: 371-424.

Moraghan, J. T., Grafton, K. (1999). "Seed-Zinc Concentration and the Zinc-Efficiency Trait in Navy Bean." Soil Sci. Soc. Am. J. 63: 918-922.

Moraghan, J.T. and Grafton, K.F. 2002. Distribution of selected elements between the seed coat and embryo of two black bean cultivars. J Plant Nutr. 25:169-176.

Muggio, B.N., M.A. Uebersax, G.L. Hosfield, and S.S. Thompson. 1985. Characterization and Bioconversion of Great Northern Bean Blancher Effluent. Journal of Food Science 50:918-921.

Murphy J. and J. Riley. 1963. A modified single solution method for the determination of phosphate in natural waters. Anal. Chim. Acta. 27:31-36.

Ng, T.J. 1990. Generation Means Analysis by Microcomputer. Hortscience 25:363-363.

Oberlas, D., Harland, B.F. 1981. Phytate content of foods: Effect on dietary zinc bioavailability. J. Amer. Dietic Assoc. 79:433.

Oberlas, D. 1983. Phytate Content in Cereals and Legumes and Methods of

Determination. Cereal Foods World 28: 352.

Ockenden, I., Falk, D.E., Lott, J.N.A. 1997. Stability of phytate in barley and beans during storage. J Agric. Food Chem. 45: 1673-1677.

Organ, M.G., Greenwood, J.S., Bewley, J.D. 1988. Phytin is synthesized in the cotyledons of germinated castor-bean seeds in response to exogenously supplied phosphate. Planta 174:513-517.

Penny, M. 1998. The role of zinc in child health. International Zinc Association, Brussels.

Raboy, V., Hudson, S.J., Dickinson, D.B. (1985). Reduced phytic acid content does not have an adverse effect on germination of soybean seeds. Plant Physiol. 79:323-325.

Raboy, V., Dickinson, D.B. (1984). "Effect of Phosphorus and Zinc Nutrition on Soybean Seed Phytic Acid and Zinc." Plant Physiol. 75: 1094-1098.

Raboy, V., Dickinson, D.B. (1993). "Phytic Acid Levels in Seed of Glycine max and G. soja as Influenced by Phosphorus Status." Crop Sci. 33: 1300-1305.

Raboy, V., Dickinson, D.B., Neuffer, M.G. (1990). "A Survey of Maize Kernel Mutants for Variation in Phytic Acid." Maydica 35: 383-390.

Raboy, V., Gerbasi, P.F., Young, K.A., Stoneberg, S.D., Pickett S.G., Bauman, A.T., Murthy, P.P.N., Sheridan, W.F., Ertl, D.S. 2000. Origin and seed phenotype of maize low phytic acid 1-1 and low phytic acid 2-1. Plant Physiol. 124:355-368.

Raboy, V., Young, K.A., Dorsch, J.A., Cook, A. 2001. Genetics and breeding of

seed phosphorus and phytic acid. J. Plant Physiol. 158:489-497.

Reddy, N.R., Pierson, M.D. 1987. Isolation and Partial Characterization of Phytic Acid-Rich Particles from Great Northern Beans (Phaseolus vulgaris L.). J. Food Sci. 52:109-112.

Reddy, N.R., Sathe, S.K., Pierson, M.D. 1988. Removal of Phytate from Great Northern Beans (Phaseolus vulgaris L.) and its Combined Density Fraction. J. Food Sci. 53:107-110.

Reddy, N.R., Pierson, M.D., Sathe, S.K., Salunkhe, D.K. 1989. Phytates in Cereals and Legumes CRC Press, Inc., Boca Raton.

Rodriguez-Burger, A.P., Mason, A., Nielsen, S.S. 1998. Use of fermented black beans combined with rice to develop a nutritious weaning food. J.Agric. Food Chem. 46:4806-4813.

Ruel, M.T., and H.E. Bouis. 1998. Plant breeding: a long-term strategy for the control of zinc deficiency in vulnerable populations. Am. J. Clin. Nutr. 68:488S-494S.

Saha, P.R., Weaver, C.M., Mason, A.C. 1994 Mineral Bioavailability in Rats from Intrinsically Labeled Whole Wheat Flour of Various Phytate Levels. J. Agri. Food Chem. 42: 2531.

Sandstrom, B., Almgren, A., Kivisto, B., Cederblad, A. 1989. Effect of Protein Level and Protein Source on Zinc Absorption in Humans. J. Nutr. 119:48.

SAS Inst., Inc. 1997. SAS Users Guide. SAS Inst., Inc., Raleigh.

Schlemmer, H.M., Jany, K. 1995. The degredation of phytic acid in legumes

prepared by different methods. European J Clinical Nutr. 49:S207-S210.

Selle, P.H., V. Ravindran, R.A. Caldwell, and W.L. Bryden. 2000. Phytate and phytase: consequences for protein utilisation. Nutrition Research Reviews 13:255-278.

Sharpley, A.N., Chapra, S.C., Wedepohl, R., Sims, J.T., Daniel, T.C., Reddy, K.R. 1994. Managing Agricultural Phosphorus for Protection of Surface Waters: Issues and Options. J. Environ. Qual. 23:437-451.

Shellie, K. C. and G. L. Hosfield (1991). "Genotype X Environmental-Effects on Food Quality of Common Bean - Resource-Efficient Testing Procedures." Journal of the American Society for Horticultural Science 116(4): 732-736.

Simmonds, N. W. 1979. Principles of crop improvement. Longman, New York.

Singh, J. P., Karamanos R.E., Stewart, J.W.B (1986). "Phosphorus-induced Zinc Deficiency in Wheat on Residual Phosphorus Plots." Agronomy Journal 78: 668-675.

Singh, J. P., Karamanos, R.E., Stewart, J.W.B. (1988). "The Mechanism of Phosphorus-Induced Zinc Deficiency in Bean." Can. J. Soil Sci. 68: 345-358.

Snapp, S. S., Lynch, J.P. (1996). "Phosphorus Distribution and Remobilization in Bean Plants as Influenced by Phosphorus Nutrition." Crop Sci. 36: 929-935.

Spencer, J.D., Allee, G.L., Sauber, T.E. 2000. Phosphorus bioavailability and

digestibility of normal and genetically modified low-phytate corn for pigs. J. Anim. Sci. 78:675-681.

Stephens, L.R., Irvine, R.F. 1990. Stepwise phosphorylation of myo-inositol leading to myo-inositol hexakisphosphate in Dictyostelium. Nature 346:580-582.

Tabekhia, M.M., Luh, B.S. 1980. Effect of Germination, Cooking, and Canning on Phosphorus and Phytate Retention in Dry Beans. J Food Sci. 45:406-408.

Teixeira, M.G., J. Guilherme, M. Guerra, D.L. de Almeida, A.P. Araujo, and A.A. Franco. 1999. Effect of seed phosphorus concentration on nodulation and growth of three common bean cultivars. Journal of Plant Nutrition 22:1599-1611.

Torre, M., A. R. Rodriguez, et al. (1991). Effects of Dietary Fiber and Phytic Acid on Mineral Availability. Critical Reviews in Food Science and Nutrition 30(1): 1-22.

Tsao, G.T., Zheng, Y., Lu, J., Gong, C.S. 1997. Adsorption of Heavy Metal Ions By Immobilized Phytic acid. Applied Biochemistry and Biotechnology 65: 731.

Turnlund, J.R., King, J.C., Keyes, W.R., Gong, B., Michel, M.C. 1984. A stable isotope study of zinc absorption in young men: effects of phytate and α-cellulose. Amer. J. Clin. Nutr. 40: 1071.

Uebersax, M.A., and C.L. Bedford. 1980. Navy Bean Processing - Effect of

Storage and Soaking Methods on Quality of Canned Beans. Michigan Agricultural Experiment Station Research Report:2-12.

Urbano, G., Lopez-Jurado, M., Aranda, P., Vidal-Valverde, C., Tenorio, E., Porres, J. 2000. The role of phytic acid in legumes: antinutrient or beneficial function? J. Physiol. Biochem. 56:283-294.

Vaintraub, I.A., Bulmaga, V.P. 1991. Effect of Phytate on the in Vitro Activity of Digestive Proteinases. J. Agr. Food Chem. 39:859-861.

Wang, X., Johnson, M.D. 1995. An isoform of 1L-myo-inositol 1-phosphate synthase (EC 5.5.1.4) from Phaseolus vulgaris (Accesion no. U38920) (PGR95-121). Plant Physiol. 110:336.

Warner, J.N. 1952. A method for estimating heritability. Agronomy J. 44:427-430.

Welch, R.M., W.A. House, S. Beebe, and Z. Cheng. 2000. Genetic selection for enhanced bioavailable levels of iron in bean (Phaseolus vulgaris L.) seeds. Journal of Agricultural and Food Chemistry 48:3576-3580.

Welch, R. M. (1993). Zinc Concentrations and Forms in Plants for Humans and Animals. Zinc in Soils and Plants. A. D. Robson. Boston, Kluwer Academic Publishers. 55.

Wilcox, J.R., G.S. Premachandra, K.A. Young, and V. Raboy. 2000. Isolation of high seed inorganic P, low-phytate soybean mutants. Crop Science 40:1601-1605.

Wise, A. 1995. Phytate and zinc bioavailability. Int. J. Food Sci and Nutr. 46: 53.

Wisker, E., R. Nagel, T.K. Tanudjaja, and W. Feldheim. 1991. Calcium,

Magnesium, Zinc, and Iron Balances in Young Women Effects of a Low Phytate Barley-Fiber Concentrate. American Journal of Clinical Nutrition 54:553-559.

World Health Organization. 1996. Trace Elements in Human Nutrition and Health. World Health Organization, Geneva.

Yoshida, K.T., Wada, T., Koyama, H., Mizobuchi-Fukuoka, R., Naito, S. 1999. Temporal and Spatial Patterns of Accumulation of the Transcript of Myo-Inositol-1-Phosphate Synthase and Phytin Containing Particles during Seed Development in Rice. Plant Physiology 119:65-72.

Zhou, J.R., Fordyce, E.J., Raboy, V., Dickinson, D.B., Wong, M.S., Burns, R.A., Erdman, J.W. 1992. Reduction of Phytic Acid in Soybean Products Improves Zinc Bioavailability in Rats. J. Nutr. 122: 2466.