IRON BIOAVAILABILITY IN LOW PHYTATE PEA

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ABSTRACT

Field pea (*Pisum sativum* L.) seeds have high nutritional value but also contain phytate which can inhibit the absorption and utilization of nutrients. Phytate is the main storage form of phosphorus in the seeds but chelates Fe, Zn and some other micronutrients and is not well digested by monogastrics. Peas with pigmented seed coats contain polyphenols which also have anti-nutritional properties. To increase the nutritional value of field pea seeds, two low phytate lines (1-150-81 and 1-2347-144) containing higher inorganic phosphorus concentration (IN-P) and lower phytate-phosphorus concentration (PA-P) than the normal phytate varieties were developed from CDC Bronco in previous research. The objectives of this research were 1) to determine the effect of genotype and environment on iron bioavailability in a set of five pea varieties differing in phytate concentration and iron concentration using *in vitro* digestion/Caco-2 cell culture bioassay; 2) to determine the effect of seed coats on iron bioavailability by testing whole seeds compared to dehulled seeds in varieties differing in seed coat pigmentation using *in vitro* digestion/Caco-2 cell culture bioassay; 3) to determine the inheritance of iron bioavailability in field pea by evaluating recombinant inbred lines differing in phytate concentration using *in vitro* digestion/Caco-2 cell culture bioassay; 4) to determine the effects of pea with the low phytate trait on body weight and hemoglobin concentration of chickens. Iron concentration (FECON) did not differ significantly between normal and low phytate varieties. Iron bioavailability (FEBIO) of the two low-phytate lines was 1.4 to 1.9 times higher than that of the three normal phytate varieties, and growing environment also had a significant effect on FEBIO. Peas with pigmented seed coats contained 7 times lower FEBIO than peas with non-pigmented seed coat. The removal of the seed coat increased the FEBIO in peas with pigmented seed coat 5 to 6 times. From previous research on PR-15 recombinant inbred lines (RILs) which were developed from a cross between low phytate line 1-2347-144 and a normal phytate variety CDC Meadow, it was found that PA-P was controlled by a single gene. FEBIO, in this study, was also found to follow a bimodal frequency distribution, characteristic of single gene

control, and it was highly correlated with PA-P in the PR-15 lines. *In vivo* studies were used to evaluate iron absorption of chickens fed with low and normal phytate pea diets. The diets containing the low-phytate pea lines had no significant effect on chicken body weight and hemoglobin level, compared with the diets containing normal phytate pea varieties. An unexpected high FECON was discovered in the diets that was traced to the ingredients of limestone and dicalcium-phosphate which likely affected the experimental results.

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LIST OF ABBREVIATIONS

AA = ascorbic acid

AAS = atomic absorption spectrophotometer

BW = body weight

CDC = Crop Development Centre

DC = 90 % of dehulled seed with 10 % of seed coat

dd H_2O = distilled deionized water

DS = dehulled seed

FEBIO = iron bioavailability

FECON = total iron concentration

Hb = hemoglobin

Hct = hematocrit

IN-P = inorganic phosphorus concentration

PA = phytate

PA-P = phytate-phosphorus concentration

ppb = parts per billion

ppm = parts per million

QTL = quantitative trait loci

SEM = standard error of the mean

SPG = Saskatchewan Pulse Growers land

WS = whole seed

1.0 General introduction

Field pea (*Pisum sativum* L.) is a cool-season legume crop which is widely used in food and feed markets (McKay et al., 2003). It is rich in protein, lysine, slowly digestible starch and fiber. However, it may also contain varying amount of anti-nutritional factors, including phytates and polyphenols (Oelke et al., 1991). Approximately 60 % of total phosphorus in crop seeds is stored as phytate, and the rest consists of inorganic phosphorus, cellular phosphorus, including phosphorous in DNA, starch and lipids, and lower inositol phosphates (Raboy et al., 2001). Phytate is a powerful inhibitor for the absorption of minerals (such as Fe, Ca, Zn) and proteins (Reddy et al., 1982; Yu et al., 2012). Because of the limited availability of phytase in humans and monogastric animals, phytate is poorly digested, which results in poor bioavailability of phosphorus and micronutrients (Erdman, 1979; Diarra et al., 2010).

During the Green Revolution, the production of crops increased rapidly, however, crop breeding based on yield often resulted in a dilution of micronutrients in crop seeds (Welch and Graham, 2002). In order to maximize the bioavailability of nutrients which were inhibited by phytate in peas, two low-phytate pea lines (1-150-81 and 1-2347-144) were developed from CDC Bronco, through chemical mutagenesis in the Crop Development Centre (CDC), University of Saskatchewan (Warkentin et al., 2012). The agronomic performance of the two low-phytate lines was tested and showed similarity with CDC Bronco, except for slightly lower seed weight and grain yield. Furthermore, the low-phytate lines presented a 60 % reduction of phytate-phosphorus concentration compared with CDC Bronco (Warkentin et al., 2012).

Iron is one of the essential elements for the body's function playing a key role in oxygen transportation and energy production (Anderson and McLaren, 2012). Iron deficiency is an important malnutrition risk, and approximately 2 billion people are affected by iron deficiency (WHO, 2002). Since phytate chelates iron, this thesis research explored the potential benefits of low phytate peas in improving iron bioavailability.

There were four hypotheses tested in this thesis: 1) Iron bioavailability is higher in low phytate field pea than in normal phytate varieties; 2) Iron bioavailability is higher in dehulled tannin-containing pea seeds than in whole seeds; 3) Iron bioavailability is segregating in a recombinant inbred line population derived from a cross between a low-phytate pea line and a normal phytate pea variety; 4) Chicken body weight and hemoglobin concentration of chicken are higher in the birds fed with low phytate pea ingredient in an iron deficient diet.

The objectives matched to each hypothesis were: 1) to determine the effect of genotype and environment on iron bioavailability in a set of five pea varieties differing in phytate concentration and iron concentration using the *in vitro* digestion/Caco-2 cell culture bioassay; 2) to determine the effect of seed coats on iron bioavailability by testing whole seeds compared to dehulled seeds in varieties differing in seed coat pigmentation using *in vitro* digestion/Caco-2 cell culture bioassay; 3) to determine whether iron bioavailability in field pea is segregating by evaluating recombinant inbred lines differing in phytate concentration using the *in vitro* digestion/Caco-2 cell culture bioassay; 4) to determine the effects of the pea with low phytate trait on body weight and hemoglobin concentration of chicken.

2.0 Literature review

2.1 Field pea origin and production, economic and agronomic value

2.1.1 Field pea origin and agronomic value

Field pea (*Pisum sativum* L.) or dry pea is an annual cool-season herbaceous legume crop (Black et al., 2006; Desai, 2004; McKay et al., 2003). Differing from garden pea which is harvested when the seeds are immature, the seeds of field peas are naturally dried to approximately 14 % moisture and consumed by humans and other animals (Black et al., 2006; Dahl et al., 2012).

The centre of origin for pea is the Fertile Crescent region, and the species appeared in central Germany six thousand years ago (Black et al., 2006). Field pea has been grown in Europe since then, and has been grown in Canada for more than 100 years (Agriculture and Agri-Food Canada, 2005; Black et al., 2006; Oelke et al., 1991; Roy et al., 2010). Field pea is a cool season legume crop. It can be grown in a wide range of soil types (Oelke et al., 1991). However, its tolerance to soils which are water-saturated or salt-affected is very poor. Hence, well-drained soils with pH 5.5 to 7.0 are ideal for pea production and these soils are quite prevalent in the agricultural region of western Canada (Agriculture and Agri-Food Canada, 2005).

Like other legume crops, the nitrogen fixation of field pea plays an important role in crop rotation systems to improve crop yields of subsequent crops and to maintain soil quality. Nitrogen fixation takes place in nodules which arise through the symbiosis of rhizobia with legume roots. In this process, N_2 is converted to ammonia by nitrogenase (Ciccolella et al., 2010; Graham, 2008; Lafond et al., 2011; Shoko et al., 2009; Wang and Daun, 2004).

2.1.2 Field pea market and production

Field pea has three cotyledon colors: yellow, green and red, and seed coat colors including non-pigmented, dun and maple (Black et al., 2006; Mendel, 1866; Oelke et al., 1991). Internationally, the yellow dry field pea has a greater market demand than green peas primarily due to a typically slightly lower price and its resistance of color change to bleach during food processing (Black et al., 2006). Meanwhile, green pea must have good natural green color in order to obtain highest grades and premium prices. The split or canned dry field pea is used directly in food markets, and the pea protein and starch are widely used in the food industry (Black et al., 2006; Lafond et al., 2011). Field pea is also used in swine and poultry feed

markets, and a smaller amount is used as birdseed or in forage markets.

The main field peas markets are in Asia and Europe (FAOSTAT, 2013). In 2010, India, China, Bangladesh and European countries were the top dry pea importing countries, and occupied approximately 80 % of the total field pea import market in 2010. Canadian domestic pea consumption was 981,000 tonnes in 2010 mainly for feed and seed to plant the next crop (Agriculture and Agri-Food Canada et al., 2005; FAOSTAT, 2013).

Since 2000, Canada has been the leading field pea producing country in the world, surpassing France, which used to be the top field pea producing country (FAOSTAT, 2013). In 2010, the global top five field pea producing countries were Canada, Russia, China, India and United States, produced 3.02, 1.22, 0.91, 0.67 and 0.64 million tonnes, respectively. In 2010, Canada was also the leading field pea exporting country, exporting 2.79 million tonnes with a value of $700 million dollars. This quantity was five times more than the No.2 exporter the USA (0.52 million tonnes). Furthermore, France and Australia were the No.3 and No.4 field pea exporting countries, with $80 million and $60 million dollars of export value each. In 2010, dry field pea occupied 2.24 % of Canadian agricultural export value, and it was the third ranked exported agricultural plant product after wheat and canola. The prairie provinces of western Canada are the major area of field pea production in Canada. Saskatchewan produces approximately 70 % of Canadian field pea production, followed by Alberta and Manitoba (Agriculture and Agri-Food Canada et al., 2005).

2.1.3 Nutritional value of field pea

Field pea contains a greater concentration of protein than cereal grains (Singh et al., 2004). Field pea seeds are also rich in slowly digestible starch, soluble and insoluble fiber, vitamins and minerals, while it contains low concentrations of sodium and fat (Oelke et al., 1991; Roy et al., 2010). Wang and Daun (2004) reported that Canadian field pea generally contains approximately 23.7 % protein, 45.5 % starch, 1.3 % fat, 2.8 % ash, 7.0 % acid detergent fiber and 9.6 % neutral detergent fiber on a dry matter basis. It is also rich in vitamins, macrominerals such as K (~1047.2 mg/100 g), P (~436.7 mg/100g), Mg (~142.4mg/100g), and other microminerals such as Fe (~59 ppm), Zn (~32ppm), Se (~457 ppb) and Ca (~77ppm) (Dahl et al., 2012; Thavarajah et al., 2010; Wang and Daun, 2004).

The nutritional level also varies among varieties of pea. Vidal-Valverde et al. (2003) reported that higher lysine and vitamin B_1 and B_2 were found in green cotyledon pea compared to yellow cotyledon pea, and the varieties with dark seed coat had lower

concentration of verbascose and sucrose, but higher phytate. Fibers in seed coat, cell wall of cotyledon, intermediate amylose proportion and the dominant C-type starch in field pea result in higher level of slowly digestible starch, compared with cereal crops (Dahl et al., 2012; Hoover et al., 2010). Due to its high level of fiber, diets with a high proportion of pea can reduce the LDL-cholesterol level which can reduce risk of heart disease and reduce blood pressure (Dahl et al., 2012; Roy et al., 2010).

Saskatchewan-produced field pea may have increased nutritional benefits compared with field pea produced in other regions. Gawalko et al. (2009) reported higher concentrations of Fe, Mg, and Mn in Canadian field pea compared to field pea produced in China. Except for North America, many people in the world have Se deficiency due to consumption of food grown on Se-deficient soils, while the soils in western Canada have abundant Se so that the field pea produced in Saskatchewan is a good natural source to meet Se requirements (Thavarajah et al., 2010).

2.2 Anti-nutritional factors and phytate

2.2.1 Anti-nutritional factors

Despite the nutritional benefits mentioned above, some field peas also contain so-called anti-nutritional factors, such as protease inhibitors, saponins, tannins and phytates, which interfere with the absorption and utilization of nutrients (Kumar, 1992; Makkar, 1993). Based on their function during the nutrient absorption, the anti-nutritional factors can be classified into 4 groups: chelating minerals (such as phytates), antivitamin, protein inhibitors (such as protease inhibitors and tannins) and inhibitors of other chemical compounds (such as saponins, nitrate and alkaloids) (Francis et al., 2001). These anti-nutritional factors are believed to play an important role in preventing seeds from the damage of environmental oxidation or insect infestation during storage (Modgil and Mehta, 1993; Xu et al., 2007).

So-called anti-nutritional factors, such as polyphenols and phytates, can have benefits or disadvantages to humans and other animals. A modest amount of anti-nutritional factors can act as antioxidants to reduce the incidence and severity of cancer and cardiovascular disease (Salvador, 2011; Schiavone et al., 2008). A chicken study showed that a diet with 0.20 % chestnut extract and additional hydrolysable tannins produced better growth performance of birds (Schiavone et al., 2008). The addition of 0.6 % by weight of a pea anti-nutritional factor concentrate, containing trypsin inhibitors and lectins isolated from the winter pea variety

Frijaune, did not affect nitrogen digestibility in the ileum of piglets (Le Guen et al., 1995). In general, these anti-nutritional factors inhibit the absorption of starch, protein and micronutrients (Carnovale et al., 1988; Dahl et al., 2012; Modgil and Mehta, 1993). According to Le Guen et al. (1995), when the concentration of the pea anti-nutritional factor concentrate was raised to approximately 3.0 %, the N digestibility of the piglets decreased. Similar observations were also found in studies of trypsin inhibitors in chickens (Gertler and Nitsan, 1970) and mice (Roy and Schneeman, 1981).

Polyphenols are found in the dry bean seed coat rather than in the cotyledon, and condensed tannin was not detectable in non-pigmented seed coats of pea seeds, but was in pigmented seed coats of pea seeds (Beninger et al., 2005; Reddy et al., 1985; Troszyńska and Ciska, 2002). Seeds with pigmented seed coat contained much greater concentrations of polyphenols than seeds with non-pigmented seed coat. In *in vitro* studies, seeds with pigmented seed coat or dark-colored fruits had a negative effect on iron bioavailability (Ariza-Nieto et al., 2007; Boato et al., 2002; Hu et al., 2006). Compared with the negative effect caused by phytate which can be reversed substantially by ascorbic acid (AA), this negative effect, caused by polyphenols, was barely reversed by AA (Engle-Stone et al., 2005).

Pea from different sources contained different anti-nutritional factors. Wang and Daun (2004) reported that overall, Canadian yellow field pea seeds on a dry matter basis contained 3.0-13.0 g/kg of phytate, 1.5-2.7 g/kg of trypsin inhibitor activity, with no tannins detectable. This differed from the Australian field pea (2.2-9.9g/kg, 0.4-2.1g/kg and 0.1-11g/kg, respectively) where dun types (pigmented seed coat) are widely grown. Phytate is relatively higher in Canadian field pea and compared to other anti-nutritional factors may be one of the major nutrition issues in Canadian field pea.

2.2.2 Phytate

2.2.2.1 History of phytate research

Phytate was first isolated by Hartig from seeds in 1855. Since this compound did not behave as protein or other well-known chemicals, it was first named as “globoid” (Reddy and Sathe, 2001; Reddy et al., 1982). Later, when inositol and phosphoric acid were harvested after the hydrolysis of these globoids, this compound was named inositol-phosphoric acid by Schulze and Winterstein (1896, as cited in Reddy et al., 2001). However, an argument was raised on the chemical structure of the inositol-phosphoric acid between Anderson and Neuberg in the next several years (Reddy and Sathe, 2001). With the development of modern

chemical analyses, the structure suggested by Anderson, was proved to be correct (Figure 2.1). Today, the most common name for this compound is phytate or *myo*-inositol hexaphosphate (IP6).

According to Raboy and Dickinson (1993, as cited in Coelho and Benedito, 2008), the available phosphate supplied by the soil determines the phytate concentration in seeds. On a dry matter basis, pea contains 3.0-13.0 g/kg (approximately 0.22 to 1.22 % of pea weight) phytate and most of the phytate is located in the cotyledons (Ariza-Nieto et al., 2007; Reddy, 2001).

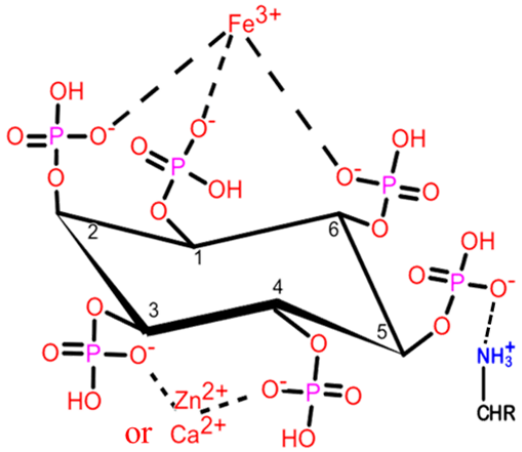

Figure 2.1. Phytate structure, the inositol binds six phosphates; the negative charge of phosphate can bind to divalent or trivalent ion (adapted from Yu et al. 2012).

2.2.2.2 Chelation properties of phytate

The pathways of phytate synthesis vary in different species, however, the synthesis of *myo*-inositol-1-P (IP1) is considered as the first step (Coelho and Benedito, 2008; Loewus, 2001). Due to level of phosphorylation of inositol and the by-products from phytate synthesis, lower phosphorylated forms IP1, IP2, IP3, IP4 and IP5 occur, as well (Coelho and Benedito, 2008; Mangels et al., 2010). Phytate (IP6) stores up to 80 % of phosphorus (P) in mature plant seeds (Bohn et al., 2008; Raboy et al., 1990).

Due to the negative charge of phosphate, phytate is a strong chelator and can bind minerals and amino acids (Figure 2.1) (Bohn et al., 2008; Lott et al., 2001; Mangels et al., 2010; Reddy et al., 1982; Yu et al., 2012). When phytate chelates a polyvalent cation, it is possible for the mineral to indirectly link phytate to proteins that have a negative charge (Lott et al., 2001). Despite the four other phosphorylated forms, only IP5 and IP6 efficiently bind minerals

such as iron (Fe), zinc (Zn), calcium (Ca) (Lott et al., 2001; Mangels et al., 2010). Only IP5 and IP6 exist in peas (Frias et al., 2003). Moreover, in mature plant seeds, IP6 is considered to be the exclusive form (Lott et al., 2001; Raboy et al., 1990).

With respect to plant, phytate supports seed germination (Bohn et al., 2008; Coelho and Benedito, 2008). During germination, phytase breaks down phytate and releases phosphate and inositol (Bohn et al., 2008; Centeno et al., 2001; EL-Mahdy and EL-Sebaiy, 1982; Kumar et al., 2010; Reddy et al., 1982). However, to human health, phytate has advantages and disadvantages. A positive advantage of phytate for humans is that it is an antioxidant. Antioxidants are considered to positively influence the incidence of cancer (carcinogens), diabetes and heart disease. Phytate can also reduce heavy metal toxicity because of its strong chelation effect and similarly its chelation properties can reduce the formation of kidney stones (Graf et al., 1987; Harland and Morris, 1995; Kumar et al., 2010). The disadvantage of phytate relates to its effect on bioavailability of nutrients (Coelho and Benedito, 2008; Kumar et al., 2010; Reddy and Sathe, 2001). If diets contain high phytate concentrations, poor nutrient bioavailability can lead to poor performance or deficiency symptoms. This is exacerbated in human and monogastric animals that have limited digestive tract phytase activity. The excretion of phytate in manure can also result in the accumulation of phosphorus in the environment and the eutrophication of water (Bohn et al., 2008). Field pea is a good source of nutrients which is widely used in food and feed markets. It could provide more available nutrition with reduced phytate concentration (Dahl et al., 2012).

Food or feed processing, such as baking, soaking and cooking, can reduce the phytate concentration (Dahl et al., 2012; EL-Mahdy and EL-Sebaiy, 1982; Kumar et al., 2010). Nevertheless, since phytate is relatively a heat stable compound, it is hard to degrade by simply cooking. Therefore, phytase is often used commercially (Kumar et al., 2010).

2.3 Micronutrients and iron bioavailability

2.3.1 Micronutrients and micronutrients deficiencies

Micronutrients, such as Zn, Se and Fe, have essential functions in humans. Adequate and diversified daily meals typically provide sufficient micronutrients (genannt Bonsmann and Hurrell, 2008). However, depending on the diet and availability of the food supply, not all people consume enough micronutrients.

Micronutrient deficiency can affect a number of areas including human growth, mental

capacity, body immune function, working capacity and overall health (Darnton-Hill et al., 2005). Approximately three billion people are affected by micronutrient deficiency, primarily Fe and Zn (Ren et al., 2007; Welch and Graham, 2004). Furthermore, among the twenty top risk factors to human health (including underweight, tobacco, and high blood pressure), Fe deficiency ranked the highest in the nutritional deficiency risk, followed by Zn deficiency and vitamin A deficiency (WHO, 2002).

2.3.2 Function of iron and iron bioavailability

Iron is the fourth most abundant element on earth (Guerinot and Yi, 1994). There are two major types of iron ion: ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}). The most common form in soil is the inorganic ferric iron compound, Fe_2O_3 (Hochmuth, 2011). This ferric iron is the most stable and abundant form of iron in the aerobic environment; correspondingly, in the anaerobic environment (such as waterlogged soil), ferric iron can be converted into ferrous iron (Guerinot and Yi, 1994; Hochmuth, 2011).

In the environment with neutral pH, ferric iron will form complexes with anions, peroxides and water which are stable and almost insoluble (10^{-17} molar), and are difficult for plants to utilize (Guerinot and Yi, 1994; King, 2013). Thirty to forty percent of soil on the earth is deficient in available iron (Guerinot and Yi, 1994; Grotz, 2006; Zhai et al., 2002). Low soil pH, high organic matter and low availability of antagonistic micronutrients (such as Zn, Ca and Mn) can increase iron availability (Hochmuth, 2011; Zhai et al., 2002).

2.3.2.1 Iron utilization and absorption in plants

Iron plays an important role in plants including the synthesis of chlorophyll, nitrogen fixation and energy transfer (Hochmuth, 2011). Iron is the third limited nutrient for the regular growth of plants after nitrogen and phosphorus (Ducklow et al., 2003; Grotz, 2006). Iron is immobile from old leaves to new leaves (Brown, 1978). Iron deficiency of plants will result in yellowed new leaves and reduced photosynthesis, growth and yield (Guerinot and Yi, 1994). The plant requires approximately 10^{-9} to 10^{-4} M/L of iron for optimal growth and the requirement of iron is continuous (Guerinot and Yi, 1994; Jia and Guo, 2010).

Iron is absorbed through mass flow, diffusion and active transport by plant roots in area approximately 1 to 4 cm behind the lateral root tips (Hochmuth, 2011; Zhai et al., 2002). However, only 3 to 9 % of iron is absorbed from soil to plant by mass flow and diffusion; therefore, the most iron is absorbed from active transport, which is an energy consuming

process (Hochmuth, 2011; Zhai et al., 2002). It is a commonly thought that the most available iron forms that plants absorb are Fe^{2+}, however the most common form in soil is Fe^{3+}. The available iron that plants absorb depends on the ability of reducing Fe^{3+} to Fe^{2+} by plant (Brown, 1978; Hochmuth, 2011).

After the Fe^{2+} is absorbed through the root plasma membrane into plant, it is oxidized back to Fe^{3+} and maintained soluble by chelation with organic acids, such as citrate, to protect plants from the toxicity of over-accumulated iron (Brown, 1978; Jia and Guo, 2010; Kim and Guerinot, 2007). The soluble iron form is later transported through the xylem to the cytoplasm of leaves, and transported by phloem to sink tissue (such as seeds) for storage (Kim and Guerinot, 2007). For legume crops, such as pea and lentil, approximately 90 % of the iron is stored in the ferritin form (Jin et al., 2009; Theil and Briat, 2004), and ferritin is stored in the embryo, not in the seed coat (Marentes and Grusak, 1998).

2.3.2.2 Iron absorption and utilization in mammals

Iron sources in diets consist of heme and non-heme types. Non-heme iron, derived from plant-based diets, exists in either ferric or ferrous form, such as ferritin, ferrous sulfate and ferric citrate (Ammerman et al., 1995; Anderson and McLaren, 2012). It supplies about 90 % of dietary iron for humans. Cereals, legumes, fruits and vegetables are the main sources. The other source is heme iron derived from meat, fish and poultry sources where it is found in hemoglobin, myoglobin and cytochromes (Ammerman et al., 1995; Anderson and McLaren, 2012; Uzel and Conrad, 1998; King, 2013).

Dietary iron is first digested in the stomach. In the low pH condition of the stomach, the non-heme and heme iron is released from ligands (Miret et al., 2003). The location of iron uptake is in the duodenal enterocytes (Conrad et al., 1999). The intestinal absorption rate of non-heme iron ranges from 2 to 20 % (Ekman and Reizenstein, 1993; Monsen, 1988; Tapiero et al., 2001). Heme iron has a significantly higher absorption rate (15 to 40 %) (Anderson and McLaren, 2012; Ekman and Reizenstein, 1993). The reason for this major difference in absorption rates is the different mechanisms of iron uptake used for non-heme and heme sources of iron.

The non-heme iron is mainly present in the intestinal lumen in a poorly soluble ferric form before it is absorbed, and non-heme iron can only be absorbed by a divalent metal transporter (DMT1) (Anderson and McLaren, 2012; King, 2013; Zimmermann and Hurrell, 2007). Since DMT1 can only accept divalent ions, only ferrous iron can be absorbed, and the

ferric iron from the non-heme iron must first be reduced into ferrous by the ferrireductases, such as duodenal cytochrome b (Dcytb), in the duodenum lumen (Anderson and McLaren, 2012). Unlike non-heme iron, once the heme iron moves into the intestinal lumen, the heme carrier protein (HCP1) can directly transport heme into the cells and then ferrous iron is released (Hoekenga et al., 2011; Miret et al., 2003).

In the duodenal enterocytes, because of the acid environment, the absorbed iron from non-heme and heme iron remains in the ferrous form. Most of the ferrous iron in the enterocytes is later carried into the blood stream, while the excess iron in the enterocytes is stored as ferritin. The iron is transferred into blood, which later binds transferrin and is transported to the liver and bone marrow for further utilization (King, 2013; Zimmermann and Hurrell, 2007).

Iron is stored in mammalian bodies as ferritin, hemoglobin, transferrin and myoglobin (Dallman, 1986; Meyers, 1996). Ferritin is a protein-iron complex and is an important storage form of iron in the body, consisting of 5 to 30 % of total body iron (Dallman, 1986; King, 2013). The main locations that store ferritin are the hepatocytes in the liver, enterocytes in the duodenum, cells in skeletal muscle and reticuloendothelial cells. Apo-ferritin is formed by 24 polypeptide subunits, and can store around 2,000 to 4,500 ferric irons with a total molecular weight of 500 kDa (Anderson and McLaren, 2012; King, 2013; Zimmermann and Hurrell, 2007). If the iron concentration is excessive, another protein, hemosiderin, will be formed to store the iron (Anderson and McLaren, 2012).

Hemoglobin, found in the erythrocytes of the blood, contains 65 to 70 % of total body iron on average (Dallman, 1986; King, 2013). Iron in the hemoglobin can help to carry oxygen from lung to other organs via the blood circulation (King, 2013). Transferrin is a transporter protein in the plasma of blood and contains about 0.1 % of total body iron (Dallman, 1986). Myoglobin occupies about 10 % of total body iron, and also participates in oxygen transport and storage (Dallman, 1986).

2.3.3 Iron deficiency, anemia and iron bioavailability

2.3.3.1 Iron deficiency and anemia

Iron deficiency can be divided into three stages which may overlap (Dallman, 1986). In the first stage, the serum ferritin has a decreasing concentration. In the second stage, the concentration of transport iron begins to decline. In the third stage, the decreased

concentration of transport iron limits the concentration of other useful iron compounds such as hemoglobin, and affects the body's physiology. The first two stages are considered as pre-anemic, and the third stage is the anemic stage (Dallman, 1986). Iron deficiency reduces the efficiency of metabolic pathways that require oxygen and the synthesis of essential enzymes for cell growth and differentiation (Anderson and McLaren, 2012; King, 2013). It leads to fatigue, poor work performance and endurance, slow development of infants, and decreased immunity (Anderson and McLaren, 2012; Bañuelos and Lin, 2008; Murray-Kolb and Beard, 2009). Iron deficiency is the most prevalent micronutrient deficiency in the world. According to the World Health Organization, iron deficiency occurs when serum ferritin level is lower than 15 µg/l (WHO and FAO, 2004). Approximately 2 billion people are iron-deficient and about 30 % of the people in the world are affected by anemia (WHO, 2002).

Depending on age, gender, diet, career, and economic status, groups of people differ in their risk to suffer iron deficiency (Anderson and McLaren, 2012; de Benoist et al., 2008; Monsen, 1988; Murray-Kolb and Beard, 2009; WHO, 2002;). Pregnant women and preschool children are most susceptible to iron deficiency (Beard, 1994; Murray-Kolb and Beard, 2009). People in developing countries are more susceptible to iron deficiency than those in developed countries. Due to the limited non-heme absorption, vegetarians have higher risk of iron deficiency. Endurance athletes and those who donate blood frequently have higher risk of iron deficiency as well (Anderson and McLaren, 2012).

2.3.3.2 Iron bioavailability

The recommended daily iron intake differs by age and gender (Health Canada, 2010): infants (0.27-11 mg/day), children (7-10 mg/day), adolescent males (8-11 mg/day), adolescent females (8-18 mg/day), pregnant females (27 mg/day) and lactating females (9-10 mg/day). According to Health Canada (2010), the tolerable upper iron intake is 40 to 45 mg/day. For vegetarians, the recommended daily iron intake is about 2 times more than for people with a diet that includes meat, because of the limited iron bioavailability of diets with only non-heme iron.

The degree to which iron is absorbed and utilized for body metabolism is called "bioavailability" (Ammerman et al., 1995). Non-heme iron, typical of staple cereals, is considered to have poor iron bioavailability (Sparks, 2012). Heme iron, typical of animal-based sources, has a relatively stable and higher bioavailability (Anderson and

McLaren, 2012). Iron bioavailability of non-heme iron is affected significantly by promoters or inhibitors in the diet (Anderson and McLaren, 2012; Jin et al., 2009). AA, meat, fish and poultry proteins are the major promoters of iron bioavailability, while phytate, tannins and Ca are the main inhibitors (Anderson and McLaren, 2012; Bañuelos and Lin, 2008; Jin et al., 2009; Kalgaonkar and Lönnerdal, 2008). AA increases non-heme iron bioavailability by converting ferric iron to ferrous iron in the lumen playing the same role as the Dcytb enzyme mentioned above (Zimmermann and Hurrell, 2007). Meat protein can release peptides with low molecular weight which compete with inhibitors of iron and make it soluble for absorption (Anderson and McLaren, 2012). Phytates and tannins can bind to iron to form insoluble complexes which lower the bioavailability of iron.

Jin et al. (2009) reported that at a certain molar ratio of Fe:promoter/inhibitor, iron bioavailability will be affected significantly, for example, AA can increase iron bioavailability by three to four fold with a Fe:AA molar ratio of 1:20. Phytate can decrease the bioavailability of non-heme iron by 80-90 %, while tannins decrease it by up to 97 %. The presence of $ZnCl_2$ (25 to 50 µmol/L) also reduced non-heme iron bioavailability in a solution containing 50 µmol/L iron in solution (Glahn et al., 2002a). Furthermore, the presence of AA in a phytate-iron source (molar ratio of Fe:PA:AA = 1:20:5 to 1:20:100) can increase the iron bioavailability (Jin et al., 2009). Although meat has high iron bioavailability, eggs and milk have lower iron bioavailability because of their non-heme iron type (Hallberg, 1981). Spinach, which commonly is considered as a good iron source, only has 1-2 % of iron bioavailability (Hallberg, 1981). Pea has only approximately 1-2 % of iron bioavailability, as well (National Research Council, 2001).

2.4 Increasing nutrient availability

There are several approaches to improving nutrient availability including consumption of diverse foods, agricultural practice, supplements, food fortification and crop biofortification (Anderson and McLaren, 2012; Zimmermann and Hurrell, 2007).

2.4.1 Agricultural practice and food processing

Increasing accumulation of micronutrients in plant seeds is the primary approach to increase the nutritional level in diets (Welch and Graham, 1999). The control of micronutrient accumulation is firstly considered as under the control of physiological processes of plants (Welch and Graham, 2004). There are several major barriers to plant micronutrient

accumulation discussed by Welch et al. (2004): sufficient micronutrients in the rhizosphere, the activity of root absorption, and the efficiency of micronutrient translocation and accumulation in the edible plant tissue. To have sufficient micronutrients in the rhizosphere, the proper application of fertilizer and soil management is required to increase the solubility of micronutrients (Bañuelos and Lin, 2008; Sims, 1986; Welch and Graham, 2004). Soil pH affects the solubility of micronutrients. Solubility of Mn and Zn complexes are dominant when pH is lower than 5.2, while Fe-oxides will be dominant above that pH (Sims, 1986).

Proper food processing can also help to control anti-nutritional factors and increase the bioavailability of nutrients. Soaking and germination can help to degrade phytate (Dahl et al., 2012; Ghavidel and Prakash, 2007; Kumar et al., 2010). Most polyphenols exist in seed coats, thus the removal of seed coats reduces the concentration of polyphenols and increases the bioavailability of nutrients, such as Fe and Ca (DellaValle et al., 2013; Ghavidel and Prakash, 2007).

2.4.2 Supplements and fortified food

In order to provide additional nutrients for consumers, supplements and fortified foods were developed. Oral iron supplements are usually in bivalent and trivalent forms. Bivalent iron supplements, such as ferrous sulfate and ferrous gluconate, provide ferrous iron to the enterocytes. However, they can cause negative effects, such as uncomfortable feeling in the stomach, if the iron is released in a short time after intake (Santiago, 2012). The ferric iron supplements, such as iron protein succinylate, have low bioavailability and are expensive. Furthermore, the absorption of iron supplements with water will be negatively affected by the intake of other bivalent ionic supplements such as Zn, whereas, the intake of both supplements will not be affected significantly if they are consumed with a meal (Whittaker, 1998). According to the National Research Council (2001), the uptake of iron supplements on an empty stomach could result in constipation, vomiting and other uncomfortable feelings.

Fortified food is the processed food with additional micronutrients such as vitamin D in milk, iodine and iron in salt, and vitamin A in oil (Ramaswami, 2007). It is a traditional approach to ensure the health of people. Ferrous sulfate is a highly soluble iron source, considered as a good food fortificant, and has a similar bioavailability of ferritin iron (Hurrell et al., 2004; Jin et al., 2009). However, $FeSO_4$ has undesirable sensory properties, such as unacceptable effects on food color, which limits its usage (Hurrell et al., 2004).

Supplements and fortified food are technical methods of providing iron but do not

address the cause of nutritional deficiency (Zimmermann and Hurrell, 2007). With respect to the developing countries, supplement and fortification are relatively expensive, and the efficiency of fortification is poor. Infants and young children who have high risk of iron deficiency have limited access to fortified food. Thus, supplements and fortified food are not considered as efficient means to ensure the health of a large population (Ramaswami, 2007).

2.4.3 Biofortification and usage

Biofortification is an agricultural approach to breed plants with enhanced nutrition in a cost-effective way (Hotz and McClafferty, 2007; Qaim et al., 2007). Unlike fortification, the obvious advantages of biofortification are that once the seeds of such nutrient-enriched crops are obtained, farmers can grow and reproduce the crops every year (Graham et al., 2001).

Tomatoes and pea (*brz*) mutants were developed to improve iron uptake by plants (Guerinot and Yi, 1994). Vitamin E, folate, Ca and Fe were enriched in the seeds of many crops including rice, maize and legumes through biofortification (Hoekenga et al., 2011; Jeong and Guerinot, 2008; Tako et al., 2009; Thavarajah and Vandenberg, 2009). “Golden rice” is a transgenic biofortified crop aimed to create a pathway to produce beta-carotene in rice endosperm, which makes vitamin A available (Jeong and Guerinot, 2008). An impact assessment has been conducted in India, and Golden rice will save one healthy life-year at a cost of US $35 (Stein, 2006).

2.4.4 Biofortified low-phytate crops

Low-phytate lines have been developed in several crops including maize (Raboy et al., 2000; Shi et al., 2003; Shi et al., 2005), barley (Larson et al., 1998; Veum et al., 2007); rice (Larson et al., 2000); soybean (Wilcox et al., 2000); common bean (Campion et al., 2009), and pea (Warkentin et al., 2012). According to Raboy (2002), in maize and barley, more than 20 different low phytate mutations were developed with a 50-95 % decrease in PA-P in the seeds. Three low phytate mutations were developed in rice with a decrease of 40-75 % in phytate concentration (Cichy and Raboy, 2009). Four independent low-phytate soybean mutations were developed with a 50-80 % reduction of phytate concentration. The decreased phytate concentration also caused an increase in IN-P (Raboy, 2002).

Recently, Warkentin et al. (2012) developed two low-phytate field pea mutations by chemical mutagenesis of the popular field pea variety CDC Bronco, called 1-150-81 and 1-2347-144. These two lines have less than half the PA-P concentration compared to CDC

Bronco and other commonly grown varieties, but have increased IN-P.

2.5 *In vitro* models for iron bioavailability

The increased concentration of nutrients in biofortified crops does not necessarily correlate with increased nutrient bioavailability for humans or animals (Hoekenga et al., 2011; Jeong and Guerinot, 2008). Bioavailability should be tested to confirm the increased absorption and utilization of nutrients from biofortified foods, as well (Krebs, 2001). This is a metabolism-based process which is affected by numerous factors such as host gender, physiologic state of host, chemical form of nutrient and food matrix (Krebs, 2001). *In vitro* models can be useful and convenient tools to test target objectives in animals and humans. Solubility, algorithms, dialyzability, gastrointestinal models, HPLC analysis and Caco-2 cell model are the main *in vitro* models to test iron bioavailability (Fairweather-Tait et al., 2005; Fairweather-Tait et al., 2007).

2.5.1 Comparison between *in vitro* models to predict iron bioavailability

Solubility

Iron solubility is a way to predict iron bioavailability by measuring iron dialyzability or diffusion through a semipermeable membrane. The advantage of this method is that it is simple, relatively cheap and easy to access (Etcheverry et al., 2012; Miller and Berner, 1989). However, the results are affected by the protein source in the diet. Furthermore, in the iron bioavailability study of Swain et al. (2003), the solubility of six commercial elemental iron powder products changed with time and pH value, compared to an *in vivo* test in rats. Iron bioavailability, predicted by solubility, is not adequate because it cannot measure the impact of other dietary constituents on absorption *in vivo* (Etcheverry et al., 2012).

Algorithms

In order to predict bioavailability, several algorithms have been developed from isotopic absorption studies. A non-heme iron bioavailability study, conducted in India, chose four algorithms to evaluate iron bioavailability (Rani et al., 2010). The correlations of iron bioavailability using different algorithms had a relatively wide range (67-85 %). It is an easier and quick prediction method after the baseline data are collected. Data from food composition tables may vary among different laboratories and the host factors are not often considered into the algorithms (Fairweather-Tait et al., 2005).

Dialyzability

In vitro dialyzability can be used to predict the digestion and release of iron from the food matrix into the lumen of the gastrointestinal tract (Miller et al., 1981). Fairweather-Tait et al. (2007) indicated that not all dialyzable iron complexes are readily available, and that small polyphenol-iron complexes which can pass the membrane have low bioavailability. Farweather-Tait et al. (2005) reported that ferritin cannot pass through the dialyzability membrane. Human and dialyzability studies of processed and unprocessed complementary infant food drew different conclusions (Mamiro et al., 2004).

The same as the solubility method, dialyzability is not adequate because it cannot measure the competition of promoters and inhibitors of nutrition in the food matrix. For example, fortified breakfast cereal is usually consumed with milk, and since milk has a high Ca concentration as an absorption competitor, the actual iron bioavailability is lower than the measurement of iron passing through the membrane (Etcheverry et al., 2012; Fairweather-Tait et al., 2007).

2.5.2 *In vitro* digestion/Caco-2 cell culture bioassay

Since the models above do not closely simulate physiological conditions, scientists are developing better models to predict bioavailability. The Caco-2 cell line was developed to replace the dialysis bag to more closely simulate the intestinal uptake of iron (Glahn et al., 1998).

2.5.2.1 Origin of Caco-2 cell

The Caco-2 cell line originated from human colon adenocarcinoma cells known as HTB-37 (Glahn, 2009). It was developed by the Sloan-Kettering Institute for cancer research (Fogh and Trempe, 1975). Today, the Caco-2 cell line is usually obtained from the American Type Culture Collection (Glahn, 2009) and maintained in Eagle's Minimum essential culture medium with Earle's balanced salt solution supplemented with 2 mM L-glutamine, 1 % non-essential amino acids, 1.0 mM sodium pyruvate and 10 % fetal bovine serum. Because of its highly characterized morphology, functional differentiation and wide range of uptake of nutrients and medicines, the Caco-2 cell line is considered to resemble human intestinal epithelial cells. Since there is no excretion during iron metabolism in cells, iron balance only depends on the iron uptake by intestinal epithelial cells.

2.5.2.2 Development of *in vitro* digestion/Caco-2 cell culture bioassay

The Caco-2 cell culture model combined with *in vitro* digestion was first published to measure iron bioavailability by Glahn et al. (1994, as cited in Glahn, 2009). This early method used the radiolabeled dialysate on the cells to replace the measurement of iron dialyzability. Glahn et al. (1998) indicated that ferritin, a protein which stores iron in the body, in the Caco-2 cell line has a high positive correlation with cell Fe content. Therefore ferritin from the Caco-2 cell line can be an effective and powerful tool to predict iron bioavailability *in vitro*. The current method of *in vitro* digestion/Caco-2 cell culture bioassay was developed by measuring ferritin. Caco-2 cells are used as a monolayer on the bottom of a culture well covered by a dialysis membrane with a 15 kDa molecular weight cut off. After pepsin digestion and pancreatin-bile digestion, food is added on the dialysis membrane to feed the Caco-2 cells. Approximately 24 hours later, Caco-2 cells are harvested for ferritin determination. ANOVA tests are used to determine whether ferritin concentration from one treatment is significantly higher than from another treatment (Tako and Glahn, 2011). Several studies were conducted to compare the *in vitro* digestion/Caco-2 cell culture bioassay with human and/or poultry tests. The *in vitro* digestion/Caco-2 cell culture bioassay can be a good predictor of iron absorption in humans (Fairweather-Tait et al., 2005; Fairweather-Tait et al., 2007; Hoekenga et al., 2011; Tako et al., 2009).

2.6 *In vivo* models for iron bioavailability

In vivo methods are also required to support *in vitro* models (Fairweather-Tait et al., 2005; Tako et al., 2010). Rodents are the main animals used for iron bioavailability measurements instead of humans as they are an efficient way to study nutrient bioavailability. Pigs and chickens are also used for bioavailability testing. Pigs are omnivorous and have similarities in gastrointestinal anatomy and physiology, and digestive and metabolic processes, compared to humans (Tako et al., 2009). However, chickens require a shorter growth period, less space and less feed compared to pigs, and are therefore a less costly model system. In addition, Fe-deficient chickens have relatively higher mRNA expression of DMT1, DcytB and ferroportin, which resembles the physiological effects of Fe deficiency of other species (Tako and Glahn, 2011) when compared with Fe-adequate chickens.

According to WHO (2013), blood hemoglobin concentration is one of the best indicators to test anemia and iron deficiency in a population. Thus, in a Fe bioavailability *in vivo* study, hemoglobin and the expression level of DMT1, DcytB and ferroportin can be good indicators

to predict iron bioavailability (Hoekenga et al., 2011; Tako and Glahn, 2011).

3.0 Experiment 1 (Phytate Study):

The effect of genotype and environment on iron bioavailability in five pea varieties differing in phytate concentration and iron concentration

3.1 Introduction

Phytate is the main storage form of phosphorus in the seeds of most crops. However, it chelates Fe, Zn and some other micronutrients and cannot be well digested by monogastrics. Low phytate mutants were developed from maize, barley, rice and other crops to increase the utilization of phosphorus by monogastrics (Larson et al., 1998; Larson et al., 2000; Raboy et al., 2000). Several iron bioavailability studies showed that the food matrix with lower phytate concentration could result in an increase of iron bioavailability (Engle-Stone et al., 2005; Haraldsson et al., 2005; Jin et al., 2009).

The low-phytate pea lines (1-150-81 and 1-2347-144) were developed from CDC Bronco. These two lines presented higher inorganic phosphorus and lower phytate-phosphorus concentrations in seeds compared with CDC Bronco (Warkentin et al., 2012). The objective of Experiment 1 was to determine the effect of genotype and environment on iron bioavailability in five pea varieties differing in phytate concentration and iron concentration using the *in vitro* digestion/Caco-2 cell culture bioassay.

3.2 Materials and Methods

3.2.1 Plant materials

Five varieties of yellow field pea were used: 1-150-81, 1-2347-144, CDC Bronco, CDC Golden and CDC Meadow. CDC Bronco, CDC Golden and CDC Meadow are popular varieties grown in western Canada, released by the CDC, University of Saskatchewan. CDC Bronco is a yellow cotyledon field pea released in 2004, characterized by lodging resistance, powdery mildew resistance and yield in western Canada (Warkentin et al., 2005). CDC Golden, a yellow cotyledon field pea released in 2003, has good performance in lodging resistance and high yield (Warkentin et al., 2004). CDC Meadow is also a yellow cotyledon field pea with good lodging resistance, powdery mildew resistance and good yield (Warkentin et al., 2007). Lines 1-150-81 and 1-2347-144 are low-phytate lines derived from CDC Bronco (described in section 2.4.4) (Warkentin et al., 2012).

All seeds were derived from POYT-PHY, 3-replicate field experiments, which were conducted by M.Sc. student Oyuntamir Delgerjav at 2009 Saskatchewan Pulse Growers land

near Saskatoon (SPG), 2009 Outlook, and 2009 and 2010 Rosthern (total of four environments) using a randomized complete block design (RCBD) (Warkentin et al., 2012). Because of heavy rainfall in 2010, the coefficient of variation for seed yield at SPG and Outlook were unacceptable, and only the Rosthern location was utilized.

3.2.2 Assessment of inorganic phosphorus concentration (IN-P)

The modified single kernel Chen's reagent method (Chen et al., 1956) was used to evaluate the IN-P of the pea samples. This assay was conducted at the Grains Innovation Laboratory at the University of Saskatchewan. The assay was conducted as follows. A single seed was placed in each well of a 48 well plate, and crushed into pieces. Then 1 ml 0.4 M hydrochloric acid (HCl) (10 µl per mg tissue) was added and the plate was placed in the 4 °C refrigerator overnight. On the second day, plates were vortexed for 10 seconds. After settling the solution for 30 minutes, 10 µl of sample extract was transferred into a 96 well microtiter plate. For each sample, 90 µl of distilled deionized H_2O (dd H_2O) and 100 µl of Chen's reagent were added. Chen's reagent for 1 plate of 96-well microtiter plate contains: 2 ml of 10 % AA (solution stocked at 4 °C), 2 ml of 2.5 % ammonium molybdate, 2 ml of 6 N sulfuric acid (H_2SO_4) and 4 ml of dd H_2O.

Six-concentration phosphorus standards were used (0, 1.55, 3.10, 4.65, 6.20, 7.75 µg/ml P), using 0, 10, 20, 30, 40, 50 µl of 1 mM dipotassium phosphate (K_2HPO_4). Ten µl 0.4 M HCl was added to each standard and dd H_2O was added to make up a volume of 100 µl, and then 100 µl of Chen's reagent was added. Samples and standards were allowed to react with the reagent for 2 hours at room temperature. The plates were read at 655 nm wave length in a xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Laboratories, Inc., ON, Canada).

Based on Chen's reagent method, which assumes 10 µl 0.4 M HCl per mg tissue, the density of seed tissue in solution was 0.1 g/ml. So the final result of IN-P is presented in µg IN-P/ g seed tissue (ppm of the seed).

3.2.3 Assessment of phytate-phosphorus concentration (PA-P)

To determine the PA-P in the seed samples, the modified Wade's reagent method (Gao et al., 2007) was used. A total of 0.05 g pea flour was weighed and added to 1 ml of 0.8 N HCl in individual centrifuge tubes, then shaken vigorously overnight at room temperature. On the second day, each sample was centrifuged at 8,000 rpm for 20 min, and 10 µl of supernatant was transferred into a new centrifuge tube with 720 µl of dd H_2O and 250 µl of modified

Wade's reagent (0.3 % sulfosalicylic acid + 0.03 % $FeCl_3 \cdot 6H_2O$, modified from Vaintraub and Lapteva, 1988, as cited in Gao et al., 2007). A 1000 ppm phytic acid stock (549.9 mg of phytic acid sodium salt hydrate in 100 ml of HCl) was diluted into 25, 50, 100, 200, 300 and 400 ppm. Ten µl of each standard was added into a new centrifuge tube, together with 720 µl of dd H_2O 250 µl of modified Wade's reagent. The reacted samples and standards were vortexed, and then 200 µl of the mixed solution was transferred into a 96 well microtiter plate. The plate was read at 490 nm using a xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Inc., ON, Canada). PA-P in the sample tissue (ppm) was calculated from the reading of spectrophotometer as follow:

$$\text{PA-P (ppm, µg/g in seed)} = \text{colorimeter reading (µg/ml in extract)} \times \frac{1\text{ ml extract}}{0.05\text{g tissue}} \quad \ldots. (3.1)$$

3.2.4 Assessment of total iron concentration (FECON)

The FECON was determined using atomic absorption spectrophotometer (AAS) in the Department of Plant Sciences, University of Saskatchewan. The digestion process was modified from the HNO_3-H_2O_2 method (Gawalko et al., 1997; Thavarajah et al., 2007) using a Vulcan 84 automatic digester (Questron Technology Corporation, ON, Canada).

A 0.5 g fine flour (<0.5 mm sieve) of ground dried peas was placed into the digestion tube, together with 6 mL nitric acid (HNO_3). Digestion tubes were placed in the preheated digester at 86 °C for 55 min. Then 3 ml 30 % hydrogen peroxide (H_2O_2) was added into each tube, and 5 minutes later, another 2 ml H_2O_2 was added. After 110 minutes, 3 ml of 6 M HCl was added to each vessel. At the end of the digestion process, the sample volume was brought to 25 ml with double distilled water. The digested samples were assessed by AAS for FECON in collaboration with Mr. Barry Goetz in the Plant Sciences Department, University of Saskatchewan.

3.2.5 Assessment of iron bioavailability (FEBIO)

The FEBIO test was conducted in the laboratory of Dr. Raymond Glahn, USDA-ARS, Ithaca, New York using an *in vitro* digestion/Caco-2 cell culture bioassay (Glahn, 2009). The standard procedure followed by Dr. Glahn's lab was utilized. A 20 g of sample (whole pea seeds, in this case) was weighed and rinsed three times with 18 megaohm water, then 60 ml of 18 megaohm water was added to result in a ratio of 1:3 (sample:water) in a beaker. The beaker, which was covered with aluminium foil, was placed in the autoclave and cooked on

liquid cycle for 30 min. After cooking, the samples were freeze dried (Labconco®, Kansas City, Missouri, USA), and then ground using a coffee grinder (Cuisinart®, Woodbridge, ON, Canada).

For the *in vitro* digestion/Caco-2 cell culture bioassay, 0.5 g cooked ground sample was weighed into a 50 ml plastic centrifuge tube, then 10 ml of solution of 140 mM sodium chloride (NaCl) and 5 mM potassium chloride (KCl) was added, and the sample was mixed to simulate digestion conditions. After adjusting sample solution to pH 2 with 0.1 M HCl, 0.5 ml pepsin solution was added to digest samples and tubes were placed in a rocking incubator for 1 hour. After incubation, the pH of the sample solution was adjusted to 5.5-6.0 with 1.0 M sodium bicarbonate ($NaHCO_3$) and 2.5 ml of pancreatin-bile solution was added to digest samples, followed by adjusting the pH to 6.9-7.0 with 1.0 M $NaHCO_3$. Both pepsin solution and pancreatin-bile solution were purified by cation exchange resin (Chelex® 100, Bio-Rad Laboratories, Inc., CA, USA).

After digestion, the Caco-2 cells were fed with 1.5 ml digested sample through a 15 kDa cut-off dialysis membrane. Then the cells were incubated to rock gently for 2 hours. Then the membrane was removed together with the digested sample solution, and the cells were placed back in the incubator without rocking for 22 hours. After the 22-hour recovery, the cells were harvested and total protein was analyzed using a colorimetric assay (DC^{TM} Protein Assay, Bio-Rad Laboratories, Inc., CA, USA), and ferritin was analyzed using an immunoradiometric assay (Fer-Iron II, Ramco Laboratories, Inc., TX, USA). Iron bioavailability of samples (ng ferritin/mg protein) from different experiment runs were standardized by a standard lentil sample included in each run.

3.2.6 Statistical analysis

The statistical analysis was conducted following Proc Mixed in SAS V9.3 (SAS Institute, Inc., 2011, NC, USA). For each trait, the mixed model of the analysis of variance (ANOVA) was established to evaluate the factors of variety, location-year (environment), and the interaction of environment and variety. In this analysis, variety and environment were considered as fixed effects, and replication was considered as a random effect. When a significant difference was determined by ANOVA, means for variety, environment or variety*environment were separated using Tukey's Mean Comparison with a significance level of $P \leq 0.05$. Finally, these results were displayed in figures using MS Excel software (Microsoft Canada Inc., Mississauga, ON, Canada).

The molar ratio of PA:Fe was calculated as it can influence FEBIO (Jin et al., 2009). In this study, PA concentration was calculated from PA-P with an assumption that all PA-P was present as IP6 in pea seeds. This test was used to estimate if the PA concentration in peas is in the range where PA concentration influences FEBIO. The molar ratio of PA:Fe was calculated as follow:

$$\text{molar ratio of PA: Fe} = \frac{n(PA)}{n(Fe)} = \frac{m(PA)}{M(PA)} \times \frac{M(Fe)}{m(Fe)} \quad \text{.....(3.2)}$$

(n= molar weight, m=mass weight, M=molar mass)

3.3 Experimental results

3.3.1 Soil and weather conditions in 2009 and 2010 experimental areas

The soil conditions (including soil type and basic nutrition status) and weather conditions (including water conditions and mean temperature) are presented in Table 3.1 and Table 3.2, respectively, for 2009 SPG (Saskatoon), 2009 Outlook, 2009 Rosthern and 2010 Rosthern.

Table 3.1. Summary of soil condition and nutrition status at 2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern.

Environment	Soil zone	N (kg/ha)	P (kg/ha)	K (kg/ha)
2009 SPG	Dark Brown	14	11	332
2009 Outlook	Dark Brown	89	60	627
2009 Rosthern	Black	15	46	629
2010 Rosthern	Black	9	46	520

Notes: From ALS Laboratory Group Agricultural Services. (From the M.Sc thesis of Oyuntamir Delgerjav).

Table 3.2. Summary of irrigation status, total precipitation and mean temperature in growing period (from May to August) at 2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern.

Environment	Irrigation status	Total Precipitation [a] (mm)	Mean Temperature[b] (ºC)
2009 SPG	NA	248	13.8
2009 Outlook	Irrigated	175	14.4
2009 Rosthern	NA	264	13.8
2010 Rosthern	NA	342	14.9

Notes: [a] based on data from Climate - Government of Canada; [b] based on data from The Weather Network; NA, not applicable;

Soil N and P concentration were relatively high at 2009 Outlook. At 2009 SPG, soil P and

K concentration were relatively low compared with the other environments. The mean temperatures of the four environments were around 14 to 15ºC. Although Outlook had the lowest precipitation during summer, the irrigation overcame the shortage of water. At Rosthern, the precipitation was almost 1.3 times more in 2010 than in 2009.

3.3.2 Analysis of variance and means

Variety and environment had significant effects on IN-P, PA-P, FECON and FEBIO (Table 3.3). The interaction between variety and environment was significant for all traits except FECON.

Table 3.3. Analysis of variance (ANOVA) table with F-values and significance levels for IN-P, PA-P, FECON, FEBIO in the five yellow field pea varieties over 4 environments (2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern).

Effect	Num DF	F-values			
		IN-P	PA-P	FECON	FEBIO
variety	4	515 ***	348 ***	3 *	128 ***
environment	3	11 **	8 ***	11 **	38 ***
environment * variety	12	7 ***	4 ***	2 ^{ns}	11 ***

Notes: IN-P – inorganic phosphorus; PA-P – phytate-phosphorus; FECON – total iron concentration; FEBIO – iron bioavailability. ns, no significant p>0.05; *, significant at p≤0.05; **, significant at p≤0.01; ***, significant at p≤0.001. Num DF, degrees of freedom.

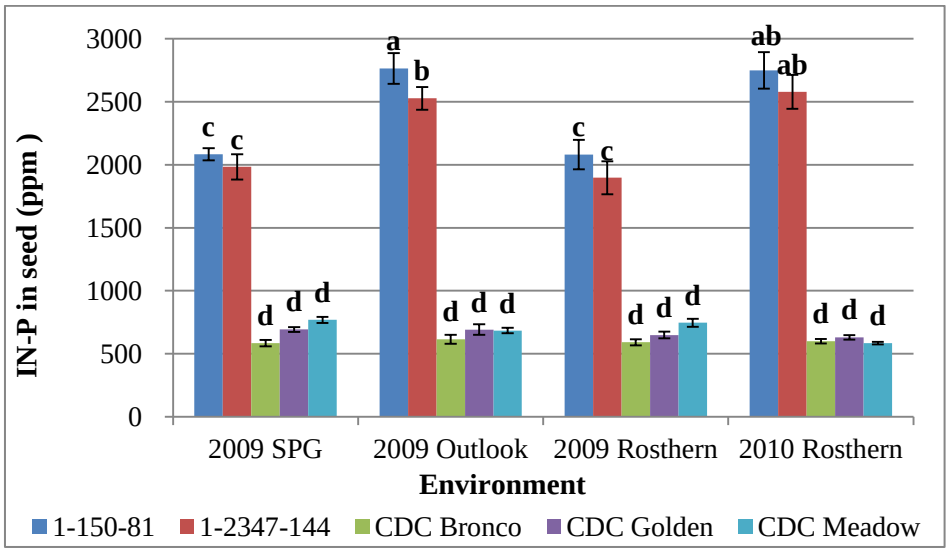

Figure 3.1. IN-P (inorganic phosphorus) of two low phytate lines (1-150-81, 1-2347-144) and three normal phytate varieties (CDC Bronco, CDC Golden and CDC Meadow) at 2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern. Letter grouping by Tukey's Mean Comparison of the interaction (variety*environment) above bars indicates significant differences (P≤0.05). Error bar shows the standard error of the mean.

For IN-P, the P-values of variety, environment and the interaction between variety and environment were less than 0.01 (Table 3.3). IN-P means among varieties and environments are presented in Figure 3.1 and Appendix Table 9.1. IN-P in 1-150-81 and 1-2347-144 (2000 to 2800 ppm) was approximately four to five times greater compared with the normal phytate varieties at each environment. Moreover, IN-P in 1-150-81 was higher than 1-2347-144. Comparing IN-P among the environments, the peas grown at 2009 Outlook and 2010 Rosthern contained greater IN-P than that at 2009 SPG and 2009 Rosthern.

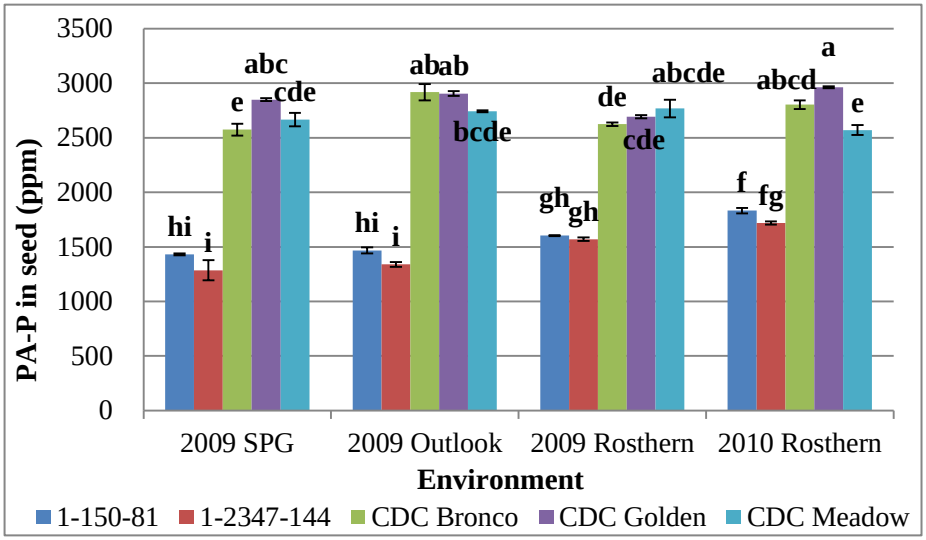

Figure 3.2. PA-P (phytate-phosphorus) of two low phytate lines (1-150-81, 1-2347-144) and three normal phytate varieties (CDC Bronco, CDC Golden and CDC Meadow) at 2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern. Letter grouping by Tukey's Mean Comparison of the interaction (variety*environment) above bars indicates significant differences ($P \leq 0.05$). Error bar shows the standard error of the mean.

For PA-P, the P-values were all less than 0.001 (Table 3.3). PA-P was significantly affected by variety, environment and their interaction. PA-P means among varieties and environments are presented in Figure 3.2 and Appendix Table 9.2. Generally, CDC Golden had the highest PA-P, while the two low phytate lines had approximately half of the PA-P compared to the normal phytate varieties for each environment. Among the three normal phytate varieties, CDC Meadow had less PA-P at 2009 Outlook and 2010 Rosthern than the other two. Among the four environments, peas grown at Rosthern in 2010 had the highest PA-P, while peas grown in 2009 contained significantly less PA-P and 2009 SPG had the least PA-P.

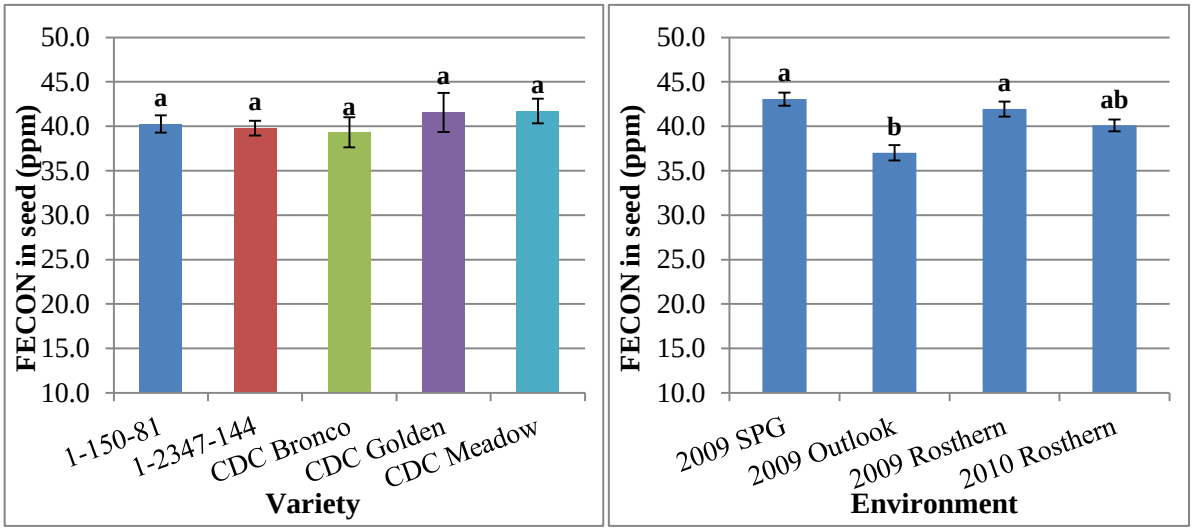

Figure 3.3. FECON (total iron concentration) of two low phytate lines (1-150-81, 1-2347-144) and three normal phytate varieties (CDC Bronco, CDC Golden and CDC Meadow); mean of four environments (left). FECON of four environments (2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern) (right). Letter grouping, by Tukey's Mean Comparison of variety and environment, respectively, indicates significant differences ($P \leq 0.05$). Error bar shows the standard error of the mean.

For FECON, the P-value of variety was 0.045 which was close to 0.05, indicating that FECON was slightly affected by variety (Table 3.3), however, according to the Tukey test, which has different assumptions and sensitivity compared to ANOVA, there were no significant differences in FECON among these varieties (Figure 3.3 and Appendix Table 9.3). The environment showed a relatively stronger effect on FECON (P-value≤0.01). The peas grown at 2009 SPG contained the highest FECON, followed by 2009 Rosthern, 2010 Rosthern and 2009 Outlook. However, the interaction between environment and variety was not significant for FECON.

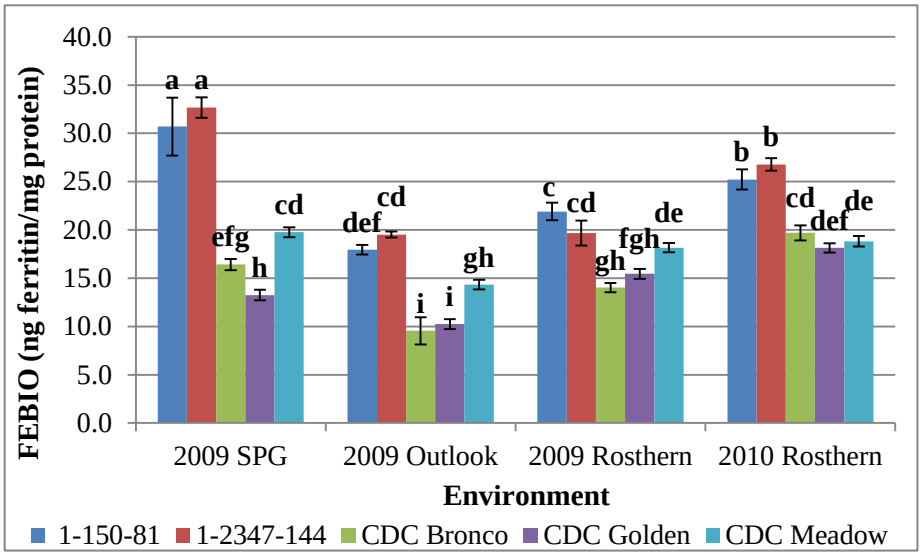

Figure 3.4. FEBIO (iron bioavailability) of two low phytate lines (1-150-81, 1-2347-144) and three normal phytate varieties (CDC Bronco, CDC Golden and CDC Meadow) at 2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern. Letter grouping by Tukey's Mean Comparison of the interaction (variety*environment) above bars indicates significant differences ($P \leq 0.05$). Error bar shows the standard error of the mean.

FEBIO was significantly affected by variety, environment and their interaction (Table 3.3). It was estimated based on the ratio of ferritin to protein concentration of the Caco-2 cells. Higher ferritin/protein ratio indicates more iron was utilized by the cells, which can be described as 'higher FEBIO'. Figure 3.4 and Appendix Table 9.4 summarized the means for FEBIO. The two low phytate lines contained similar FEBIO at 2009 SPG, 2009 Rosthern and 2010 Rosthern. Generally, the two low phytate lines had 1.5 to 2 times higher FEBIO than the three normal phytate varieties. Among the normal phytate varieties, FEBIO of CDC Meadow was significantly higher than CDC Golden at 2009 SPG, 2009 Outlook, whereas they contained similar FEBIO at 2009 Rosthern and 2010 Rosthern. At 2009 Rosthern, CDC Meadow had similar FEBIO compared with 1-2347-144. Peas grown at 2010 Rosthern and 2009 SPG had significantly greater FEBIO than at the other two environments.

Table 3.4. Molar ratio of PA:Fe present in the pea seeds of two low phytate lines (1-150-81, 1-2347-144) and three normal phytate varieties (CDC Bronco, CDC Golden and CDC Meadow) at 2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern.

		ppm in seed			Molar ratio
Environment	Variety	PA-P	Estimated PA concentration	FECON	n(PA):n(Fe)
2009 SPG	1-150-81	1432	5080	42.7	10:1
2009 SPG	1-2347-144	1285	4561	40.6	10:1
2009 SPG	CDC Bronco	2574	9134	43.1	18:1
2009 SPG	CDC Golden	2849	10108	44.7	19:1
2009 SPG	CDC Meadow	2667	9464	44.2	18:1
2009 Outlook	1-150-81	1468	5208	39.3	11:1
2009 Outlook	1-2347-144	1339	4751	37.4	11:1
2009 Outlook	CDC Bronco	2918	10353	34.9	25:1
2009 Outlook	CDC Golden	2905	10309	35.2	25:1
2009 Outlook	CDC Meadow	2743	9734	38.4	22:1
2009 Rosthern	1-150-81	1603	5690	40.9	12:1
2009 Rosthern	1-2347-144	1571	5573	40.9	12:1
2009 Rosthern	CDC Bronco	2624	9310	40.0	20:1
2009 Rosthern	CDC Golden	2694	9559	44.2	18:1
2009 Rosthern	CDC Meadow	2768	9821	43.7	19:1
2010 Rosthern	1-150-81	1832	6500	38.1	14:1
2010 Rosthern	1-2347-144	1719	6100	40.4	13:1
2010 Rosthern	CDC Bronco	2804	9950	39.4	21:1
2010 Rosthern	CDC Golden	2963	10512	42.1	21:1
2010 Rosthern	CDC Meadow	2570	9120	40.5	19:1

Notes: PA-P – phytate-phosphorus; FECON – total iron concentration; FEBIO – iron bioavailability. PA – phytate, estimated from PA-P with an assumption that all PA-P was in IP6 form

The estimated phytate concentration in seeds is presented along with the molar ratio of phytate to total iron (PA:Fe) of each variety in each environment (Table 3.4). The estimated phytate concentration was calculated from PA-P with the assumption that all PA-P comes from IP6. In this experiment, at 2009 SPG, the estimated PA:Fe ratios of the two low phytate lines were 10:1, while the ratios of the normal phytate varieties were all close to 20:1. At 2009 Outlook, the PA: Fe ratios ranged from 11:1 to 25:1, and CDC Bronco and CDC Golden had almost 2.6 times higher PA:Fe ratios compared to low phytate lines. At 2009 Rosthern, the PA:Fe ratio ranged from 12:1 to 20:1. At 2010 Rosthern, the PA:Fe ratios ranged from 13:1 to 21:1. Summarily, over the four environments, the low phytate lines had PA:Fe molar ratio

from 10:1 to 14:1, while the normal phytate varieties had 1.8 to 2.6 times higher PA:Fe molar ratio (ranging from 18:1 to 25:1) compared to the low phytate lines, and CDC Meadow had slightly lower PA:Fe ratio compared with CDC Bronco and CDC Golden.

3.4 Discussion

Breeding for low phytate is an efficient biofortification technique to increase IN-P in staple crops. The low phytate trait had a significant effect on increasing IN-P and decreasing PA-P in pea seeds. Warkentin et al. (2012) demonstrated that there was no significant difference in total phosphorus concentration of these varieties. In this study, due to the 2-year storage of seeds, IN-P and PA-P of these varieties were re-tested and were confirmed to be similar. Since total phosphorus in the seeds can be considered as the sum of IN-P and PA-P overall (Thavarajah et al., 2013), the calculated total phosphorus in this study obtained similar results compared to the total phosphorus data described in the M.Sc. thesis of Delgerjav (2012). Low-phytate lines were also developed from several other crops including maize, barley, rice, soybean, and common bean (Campion et al., 2009; Larson et al., 1998; Larson et al., 2000; Raboy et al., 2000; Shi et al., 2003; Shi et al., 2005; Veum et al., 2007; Wilcox et al., 2000). Their studies also showed that these low phytate mutations resulted in a reduction of 40-95 % in PA-P in seeds, and an increase of IN-P.

The precipitation during the growing season in 2010 Rosthern was nearly 100 mm more than at the other environments (Table 3.2). Wet soil conditions often cause an increased availability of nutrients, including P (Shapiro, 1958; Weber et al., 2010). This might explain why IN-P and PA-P were higher at 2010 Rosthern than at 2009 Rosthern. In a separate study using these low phytate field pea lines, Thavarajah et al. (2013) reported that total phosphorus concentration in seeds increased as P fertilizer rates increased; however, P fertilizer rates did not affect iron concentration in seeds.

The FECON was significantly affected by the environment in which they were grown. Meanwhile, the P-value of variety in ANOVA test was 0.045, indicating the variety had relatively small effect on FECON. Ariza-Nieto et al. (2007) and DellaValle et al. (2013) working with bean and lentil, respectively, reported that seeds harvested from the same location differed in FECON in different varieties. Common bean seeds harvested from acid soil contained 25 % higher FECON than from calcareous soil (Moraghan et al., 2002). Therefore it can also be influenced by soil type, weather and nutrients in soil. Although pea varieties differed significantly in FECON in this study, it did not show a consistent pattern

among varieties at each environment which meant the effect of environment was greater than the effect of variety.

The FEBIO is influenced by the type of iron stored in the food. Heme iron, found in animal sources such as hemoglobin, has relatively high and stable FEBIO. However, non-heme iron, found in plant source such as ferrous sulfate and ferric citrate, has low and unstable FEBIO but provides most of the dietary iron (Ammerman et al., 1995; King, 2013; Theil and Briat, 2004). The bioavailability of non-heme iron can be easily affected by inhibitors (such as phytates and polyphenols) and promoters (such as fish, meat, and AA). Although FECON is positively correlated with the concentration of Zn or Ca in seeds, with respect to bioavailability, these minerals are competitors (Welch et al., 2000). It is reasonable to increase FEBIO in staple food to address iron deficiency issue worldwide.

Low phytate is a biofortification technique for staple crops to increase FEBIO, although FEBIO is controlled by many factors in seeds and food matrix, as well as soil and weather conditions (Anderson and McLaren, 2012; Bañuelos et al., 2008; Jin et al., 2009; Kalgaonkar et al., 2008). In this study, FEBIO, unlike FECON, displayed a consistent pattern that it was 1.5 to 2 times higher in the low phytate lines with 50 % lower PA-P than normal phytate varieties across all environments. Similarly, according to Aluru et al. (2011), low phytate maize line (*lpa1-1*) was developed with 60 % lower phytate concentration and 1.5 times higher FEBIO than the normal phytate variety A188.

For the varieties differing in phytate concentration, the molar ratio of PA:Fe might have the biggest effect on FEBIO. Glahn et al. (2002a) demonstrated that in the food matrix only using $FeCl_3$ as iron source, FEBIO decreased as PA:Fe increased from 0:1 to 10:1. However, the PA concentration had no significant effect on FEBIO when the PA:Fe molar ratio rose above 10:1. This may help to explain the smaller difference in FEBIO between the low and normal phytate varieties at 2009 Rosthern and 2010 Rosthern where all varieties contained higher than 10:1 molar ratio of PA:Fe.

The other reason for the smaller difference in FEBIO between low phytate lines and normal phytate varieties at 2009 Rosthern and 2010 Rosthern is the soil nutrient status and soil type. The amount of available nutrients in the soil affects the amount of nutrients that crops absorb and utilize (Bañuelos and Lin, 2008; Sims, 1986; Welch and Graham, 2004). Soil iron concentration was not tested in this study. The soil color can determine the iron status and other nutrition status in soil (Kafoor, 2013).

3.5 **Conclusions**

The two low phytate lines contained around four to five times higher IN-P and nearly 50 % reduction of PA-P compared with the normal phytate varieties. The low phytate trait did not influence the FECON, but resulted in a significant increase in FEBIO. The low phytate pea lines contained around 1.5 to 2 times higher FEBIO than the normal phytate pea varieties. All four traits (IN-P, PA-P, FECON and FEBIO) of the pea seeds were significantly affected by environment. However, regarding FEBIO, the low phytate trait had a greater effect than environment. Hence, the low phytate trait is useful to increase FEBIO in peas.

4.0 Experiment 2 (Seed Coat Study):

The effect of seed coats on iron bioavailability in pea varieties differing in seed coat pigmentation

4.1 Introduction

Polyphenols inhibit the bioavailability of micronutrients (Carnovale et al., 1988; Dahl et al., 2012; Modgil and Mehta, 1993). The main dietary polyphenols are phenolic acids and flavonoids (Scalbert and Williamson, 2000). The major sources of dietary polyphenols are fruits, vegetables, legumes and cereals. In peas and beans, most polyphenols exist in the seed coat; in contrast, phytate is located in the cotyledons (Ariza-Nieto et al., 2007; Beninger et al., 2005; Dahl et al., 2012). Dehulling seeds can remove most of the polyphenols. In lentils and beans, the dehulled seeds have greater iron bioavailability than the whole seeds (Ariza-Nieto et al., 2007; DellaValle et al., 2013).

The seed coat color of legumes is determined by polyphenol composition (Feenstra, 1960; Hu et al., 2006). Pigmented seeds and fruits contain much greater concentration of polyphenols than non-pigmented ones. The bioavailability of nutrients was significantly higher in non-pigmented seeds or fruits (Boato et al., 2002; Tako and Glahn, 2011). Unlike the case for phytate, the iron bioavailability decreased by polyphenols cannot be reversed and enhanced by AA (Ariza-Nieto et al., 2007; Hu et al., 2006; Jin et al., 2009; Troszyńska and Ciska, 2002).

The objective of this experiment was to determine the effect of seed coats on iron bioavailability by testing whole seeds compared to dehulled seeds in varieties differing in seed coat pigmentation using *in vitro* digestion/Caco-2 cell culture bioassay.

4.2 Materials and methods

Pea seed samples of five varieties (1-2347-144, CDC Bronco, CDC Meadow, CDC Rocket and 40-10) were from POYT-PHY, 3-replicate field trials, in 2009 and 2010 at Rosthern. 1-2347-144, CDC Bronco and CDC Meadow have non-pigmented seed coats, yellow cotyledons and a round seed shape (Warkentin et al., 2005; Warkentin et al., 2007; Warkentin et al., 2012). CDC Rocket has maple-type, speckled dark pigmented seed coat, yellow cotyledons and a moderately cubed seed shape (Jin et al., 2012; Marles et al., 2013). 1-2347-144, CDC Bronco, CDC Meadow and CDC Rocket were developed by the Crop Development Centre, University of Saskatchewan. Variety 40-10, was developed in Germany,

is grown as a forage pea in western Canada, and it has speckled dark pigmented seed coats, yellow cotyledons and a small seed size (Warkentin et al., 2009).

Three treatments were examined: 1) whole seeds, 2) dehulled seeds, and 3) dehulled seed + 10 % of seed coat added back. The seed coats were removed using Satake TM-05 Grain Test mill (Satake Engineering Co., Ltd., Japan). The percentage of dehulled seeds and seed coat was recorded and calculated based on the weight of the whole seeds. Total iron concentration and iron bioavailability tests were conducted, following the methods described in sections 3.2.4 to 3.2.5. Iron bioavailability of samples (ng ferritin/mg protein) from different experiment runs were standardized by lentil sample in each run.

ANOVA, following Proc Mixed procedure, was conducted using SAS ® V9.3 (SAS Institute Inc. 2011, Cary, NC) to determine the effect of variety, year and their interaction on seed coat weight, FECON and FEBIO. Variety and or year were considered as fixed effects, and replication was considered as a random effect. When a significant difference was determined by ANOVA, means for variety, year or variety*year were separated using Tukey's Mean Comparison with a significance level at $P \leq 0.05$. The graphs were prepared in MS Excel software (Microsoft Canada Inc., Mississauga, ON, Canada).

4.3 Experimental results

Seed coat weight (as a % of the whole seeds) was affected by variety and year, but not their interaction (Table 4.1). Weight distribution (%) of the seed coat in whole seed is shown in Table 4.2, together with the letter grouping by Tukey's Mean Comparison. The seed coat occupied approximately 4.0 to 8.7 % of the weight of the whole seeds. The percentage weight of seed coat was higher in 40-10 compared with the other varieties. Seeds had greater seed coat weight in 2010 Rosthern than 2009 Rosthern.

Table 4.1. ANOVA table with F-values and significance levels for seed coat weight (% of the whole seed) at Rosthern in 2009 and 2010 of five pea varieties 1-2347-144, CDC Bronco, CDC Meadow, CDC Rocket and 40-10.

Effect	Num DF	F-values
variety	4	15.9***
year	1	19.8***
year*variety	4	1.7^{ns}

Notes: ns, no significant $p>0.05$; ***, significant at $p \leq 0.001$. DF, degrees of freedom.

Table 4.2. Weight distribution (%) of the seed coat in seeds of five pea varieties 1-2347-144, CDC Bronco, CDC Meadow, CDC Rocket and 40-10 at Rosthern in 2009 and 2010.

	Year			
Variety	2009	2010	Variety Mean	SEM
1-2347-144	5.6 de	6.4 bcd	6.0 bc	0.4
CDC Bronco	4.9 ef	5.3 def	5.1 cd	0.2
CDC Meadow	4.0 f	6.0 cde	5.0 d	1.0
CDC Rocket	4.9 ef	7.3 bc	6.1 b	1.2
40-10	7.7 ab	8.7 a	8.2 a	0.5
Year Mean	5.4 b	6.7 a		

Note: Means for variety, year and their interaction were separated by Tukey's Mean Comparison with a significance level at P≤0.05. SEM, standard error of the mean.

The FECON was differed significantly among varieties, but it was affected neither by year nor by the interaction between year and variety (Table 4.3). Variety 40-10 contained higher FECON than the other varieties. The FECON of CDC Rocket did not differ from that of the other three varieties with non-pigmented seed coat (Figure 4.1). CDC Bronco had the lowest FECON.

Table 4.3. ANOVA table for FECON at Rosthern in 2009 and 2010 of five pea varieties 1-2347-144, CDC Bronco, CDC Meadow, CDC Rocket and 40-10.

Effect	Num DF	F-values
variety	4	39.9***
year	1	6.5^{ns}
year*variety	4	1.8^{ns}

Notes: FECON – total iron concentration; ns, no significant p>0.05; ***, significant at p≤0.001. DF, degrees of freedom.

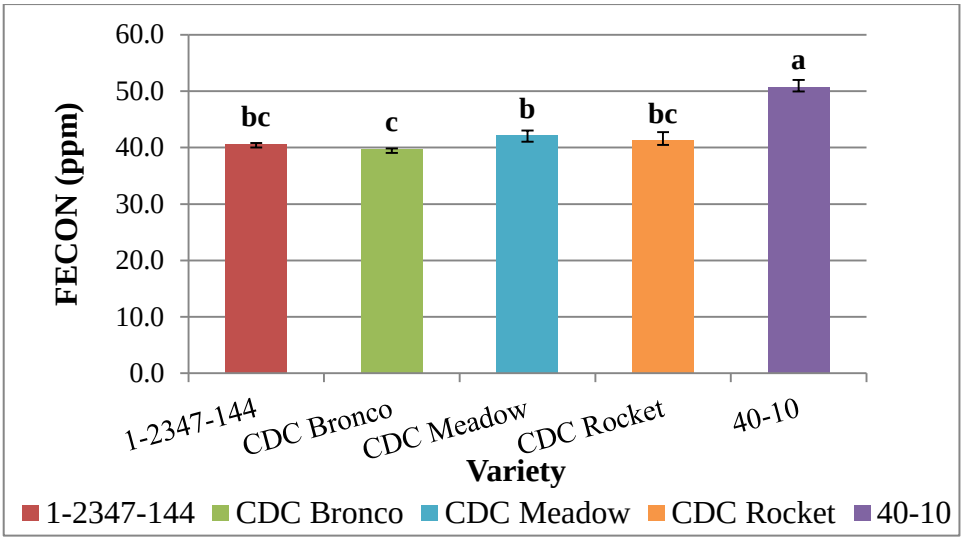

Figure 4.1. The average FECON of whole seed of 1-2347-144, CDC Bronco, CDC Meadow, CDC Rocket and 40-10 at 2009 and 2010 Rosthern. Letters above bars indicates significant differences (P <0.05). Error bar shows the standard error of the mean.

The FEBIO was highly affected by the variety and the treatment (Table 4.4). Environment, the interaction between environment and variety, the interaction between environment and treatment, and the interaction between variety and treatment had no significant effects on FEBIO.

Table 4.4. ANOVA table with F-values and significance levels for FEBIO at Rosthern in 2009 and 2010 with three treatments (WS – whole seed, DS – dehulled seed, DC – (90 %) dehulled seed with (10 %) seed coat).

Effect	Num DF	F-values
variety (G)	4	45.4***
treatment (T)	2	22.6***
environment (E)	1	3.2^{ns}
G*E	4	0.5^{ns}
G*T	8	2.7^{ns}
E*T	2	3.2^{ns}

Notes: FEBIO – iron bioavailability; ns, no significant p>0.05; ***, significant at p≤0.001. DF, degrees of freedom.

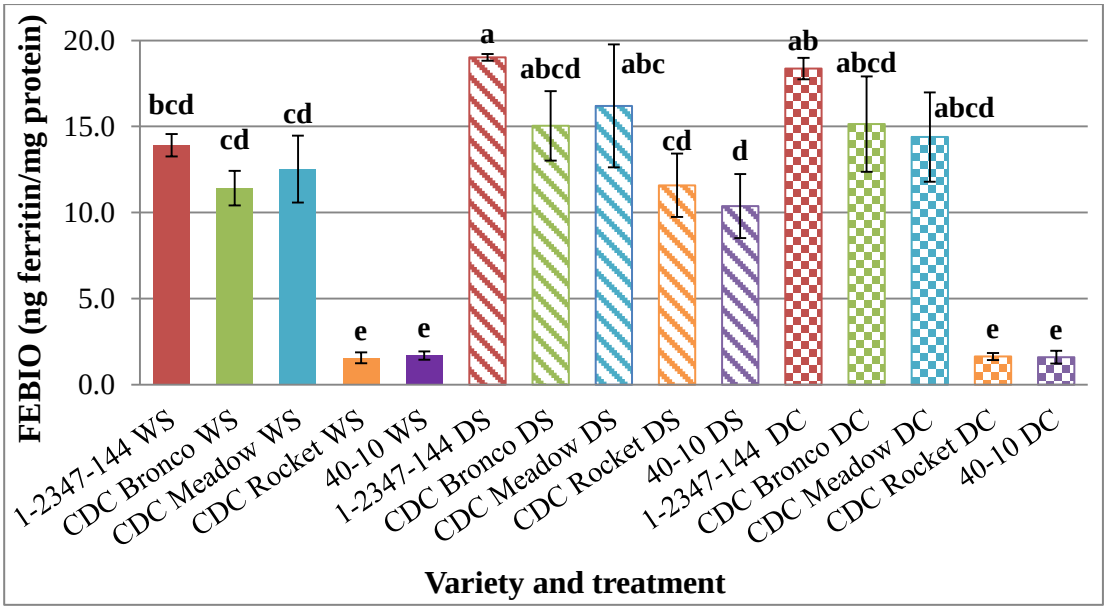

Figure 4.2. FEBIO of five varieties (1-2347-144, CDC Bronco, CDC Meadow, CDC Rocket, 40-10) with three treatments (WS, whole seed; DS, dehulled seed; DC, (90 %) dehulled seed with (10 %) seed coat) in 2009 and 2010 Rosthern. Letters above bars indicates significant differences (P <0.05). Error bar shows the standard error of the mean.

When evaluating the whole seeds (WS), the varieties with pigmented seed coats (CDC Rocket and 40-10) had seven times lower iron bioavailability than non-pigmented varieties, despite 40-10 containing higher FECON than the other pea varieties (Figure 4.2). When the seed coat was removed, the iron bioavailability of CDC Rocket (DS) and 40-10 (DS) increased five to six times, compared to WS treatment. However, when 10 % of the seed coat was added back into the dehulled seed flour (DC), the iron bioavailability decreased substantially again for CDC Rocket and 40-10.

4.4 Discussion

CDC Rocket and 40-10 had greater seed coat weight than the three non-pigmented varieties. Chen and Heneen (1992) concluded from several rapeseed studies that non-pigmented seed coats were thinner than pigmented seed coats. This result might also be due to the moderately cubed seed shape of CDC Rocket and small seed size of 40-10 which increased the surface area of the seed or increased the ratio of seed coat: whole seed. In contrast, the other three varieties had round seed and larger seed size.

Ariza-Nieto et al. (2007) reported that 79 to 95 % of iron in common bean seeds are located in the cotyledons, 1.1 to 3.6 % in the embryo axis, and 4.1 to 26.4 % in the seed coat. Marentes and Grusak (1998) reported, almost all iron (42 to 92 %) was stored as ferritin in the cotyledons and embryo axis in pea seeds. Thus, the removal of seed coat would not cause

a big loss of iron in pea seeds.

FEBIO is associated with iron concentration, form of iron complexes, and the presence of other promoters and inhibitors in the food matrix (Ariza-Nieto et al., 2007). FECON, in this case, had very limited influence on FEBIO, compared to the presence or absence of polyphenols. Although 40-10 whole seeds had 25 % more FECON than varieties with non-pigmented seed coat, its FEBIO was only 15 to 20 % of that of the varieties with non-pigmented seed coat. Ariza-Nieto et al. (2007) also reported that the bean variety, G19833 with yellow and red-mottled seed coats, had high FECON in whole seeds, however, its FEBIO was lower than other varieties.

Polyphenols, such as flavonol glycosides and tannins, contribute to the seed coat color of peas and mostly appear in the seed coat (Feenstra, 1960; Marles et al., 2013). Hu et al. (2006) showed that the polyphenol, kaempferol in the pigmented bean seed coat inhibited FEBIO. Boato et al. (2002) using an *in vitro* study showed that red grape juice can inhibit FEBIO, while white grape juice enhanced FEBIO. Tako and Glahn (2011) also reported that FEBIO was promoted by white bean but inhibited by red bean, using both *in vitro* and *in vivo* studies. The removal of seed coats can decrease the amount of polyphenols in seeds. When the pigmented seed coat of lentils and beans were removed, the FEBIO increased significantly as demonstrated using the *in vitro* digestion/Caco-2 cell culture bioassay (Ariza-Nieto et al., 2007; DellaValle et al., 2013).

Polyphenols and phytate are both considered to be anti-nutritional factors, but their effects on FEBIO are different. In this study, the difference of FEBIO between low and normal phytate varieties (1.1 to 1.2 times higher) was much smaller than FEBIO between varieties with non-pigmented and pigmented seed coat (7 times higher) in 2009 and 2010 Rosthern, indicating the polyphenols had stronger effect than phytate in these varieties. Jin et al. (2009) reported that the lowered FEBIO caused by the presence of phytate in food can be enhanced greatly by the addition of AA. However, the lowered FEBIO caused by polyphenols cannot be reversed and enhanced by AA.

4.5 Conclusion

The removal of the seed coat of pigmented pea varieties caused a significant increase in FEBIO. When 10 % of the pigmented seed coat was added back, the FEBIO decreased to the previous level. Compared to FECON, polyphenol concentration had greater influence on

FEBIO.

Although polyphenols have benefits in reducing the risk of cardiovascular disease, they tend to cause reduction in FEBIO. CDC Rocket is a maple pea variety and this market class is mainly used in bird seed mixtures. Variety 40-10 is a forage pea variety and thus the biomass is used for feeding ruminant animals. Thus, neither is intended for human consumption markets as is the case for the other three varieties in the study. In order to reduce iron deficiency, it would be appropriate to consume non-pigmented pea, and this is the prevalent choice in the market.

5.0 Experiment 3 (Inheritance Study):

The inheritance of iron bioavailability in field pea recombinant inbred lines differing in phytate concentration

5.1 Introduction

Iron is an essential element to humans and animals. Iron deficiency is the top malnutrition problem in the world (Welch and Graham, 2004). Iron concentration, anti-nutritional factors, and promoters (AA or meat) in the food can influence the iron absorption and utilization by humans and other animals (Anderson and McLaren, 2012; Jin et al., 2009). In order to improve the utilization of nutrients, two low phytate field pea lines were developed at the Crop Development Centre (CDC), University of Saskatchewan with a 50 % of reduction of phytate-phosphorus concentration compared to normal phytate varieties (Warkentin et al., 2012). It was confirmed that foods containing more phytate have lower iron bioavailability (Glahn et al., 2002a) and these low phytate pea lines have increased iron bioavailability over normal phytate peas (Experiment 1 Phytate Study).

The PR-15 recombinant inbred line (RIL) population was developed by crossing one of the low phytate lines (1-2347-144) and a normal phytate pea variety (CDC Meadow). Rehman et al. (2012) reported that the low phytate trait in lines 1-2347-144 and 1-150-81 is controlled by single gene. The objective of Experiment 3 was to determine inheritance of iron bioavailability in field pea by evaluating recombinant inbred lines differing in phytate concentration using the *in vitro* digestion/Caco-2 cell culture bioassay.

5.2 Materials and Method

5.2.1 Plant materials

The low phytate line 1-2347-144 and a normal phytate variety CDC Meadow were crossed and F_8 RILs (163 lines) were developed using single seed descent by Dr. Aziz Rehman and Mr. Arun Shunmugam in the Plant Sciences Department, University of Saskatchewan. The RILs and parents were grown in a single replicate field nursery at the Sutherland (Saskatoon) nursery in 2011, and in 2-replicate, 2-location (Sutherland and Rosthern) field experiments in 2012 and 2013 by Mr. Shunmugam as part of his PhD research. Due to the limited experimental capacity for the *in vitro* digestion/Caco-2 cell culture bioassay at USDA-ARS, Ithaca, New York, only a subset (80 out of 163) of the RILs from the 2012 Rosthern location (with 2 replications) was selected for evaluation of iron bioavailability.

The same modified Wade's reagent method, mentioned in section 3.2.3, was used to evaluate the PA-P concentration for the 163 RILs in 2011 Sutherland (single replication), 2012 Sutherland and 2012 Rosthern (two replications each). An estimation of the cut-off values of PA-P was conducted to separate the RILs into 3 levels (high PA-P, low PA-P and intermediate) within each 5 location-year-replication. RILs with consistent PA-P were selected. After the selection of RILs based on consistent PA-P, if the number of lines was more than 80 (in fact, 93 lines were obtained), a random selection was conducted to obtain 80 lines using Minitab (Minitab Inc., State College, PA, USA). Hence, 80 lines*2 replications in 2012 Rosthern were selected for the iron bioavailability test.

5.2.2 Assessment of FECON and FEBIO

FECON was conducted follow the procedure in section 3.2.4, and FEBIO test was conducted in the lab of Dr. Glahn using the procedure described in section 3.2.5., except the method to test ferritin followed an enzyme immunoassay procedure (Spectro Ferritin, Ramco Laboratories Inc., Stafford, TX, USA). This new method avoided the use of radioactive isotopes making it safer and quicker. The same protein assay was used; however, the reading was conducted using an Epoch microplate Spectrophotometer (BioTek®, Winooski, VT, USA). FEBIO of samples from different experiment runs was standardized by the percentage (%) to 1-2347-144 in each run.

5.2.3 Statistical analysis

In the PA-P test, the frequency distribution of PA-P was drawn using MS Excel (Microsoft Canada Inc., Mississauga, ON, Canada) to estimate if the distribution was affected by the selection of RILs. The unit in this test was the ppm in the extraction which could be converted into ppm in the seed by multiplying by 20.

In the iron bioavailability test, due to the relatively large sample size and the laboratory capacity to conduct the assay, the test was separated into 8 experimental runs (4 experimental runs of iron bioavailability for each replication in 2012 Rosthern). Since the activity of Caco-2 cells in each experimental run might be slightly different, line 1-2347-144 was repeated in each run as a standard. Thus, the final unit to represent iron bioavailability is described as the percentage of iron bioavailability in individual RILs compared to 1-2347-144 in this experiment (% to 1-2347-144), as is the typical procedure in the Glahn's laboratory.

An ANOVA test was carried out using SAS ® V9.3 (SAS Institute Inc. 2011, Cary, NC),

using the Proc Mixed procedure, in which entry (lines) was considered a fixed effect and replication was considered a random effect. The average of iron bioavailability (% to 1-2347-144) was calculated from the two replications in 2012 Rosthern, which was later described in a frequency distribution graph by MS Excel software (Microsoft Canada Inc., Mississauga, ON, Canada).

5.3 Experimental results

5.3.1 Assessment of FECON, PA-P and selection for FEBIO test

Figure 5.1 shows the frequency distribution for PA-P based on the average of PA-P of the two locations and two replications in 2012 for the RILs before (163 RILs, graph A) and after (80 RILs, graph B) selection. Both of the frequency distributions were bimodal, as expected from a RIL population segregating for a single gene (Rehman et al., 2012). Therefore, the selection did not substantially affect the frequency distribution of the PA-P trait.

5.3.2 Inheritance of FEBIO

The PR-15 lines had a significant effect on both FECON and FEBIO (P-value < 0.001) (Table 5.1). FEBIO (% to 1-2347-144) of RILs from these two replications was averaged. The population median was 93.8 %, CDC Meadow was 84.1 % and 1-2347-144 was 100 %. The distribution of FEBIO for the RILs was continuous but followed a bimodal pattern with the first peak in the range of 72.5-87.5 % and the second peak at 102.5-117.5 % (Figure 5.2).

Table 5.1. ANOVA table of the FEBIO and FECON (% to 1-2347-144) for the subset of PR-15 with 80 RILs in 2012 Rosthern with two replications.

Trait	Num DF	F Value
FEBIO	79	3.9 ***
FECON	79	2.1 ***

Notes: ***, significant at p≤0.001. DF, degrees of freedom.

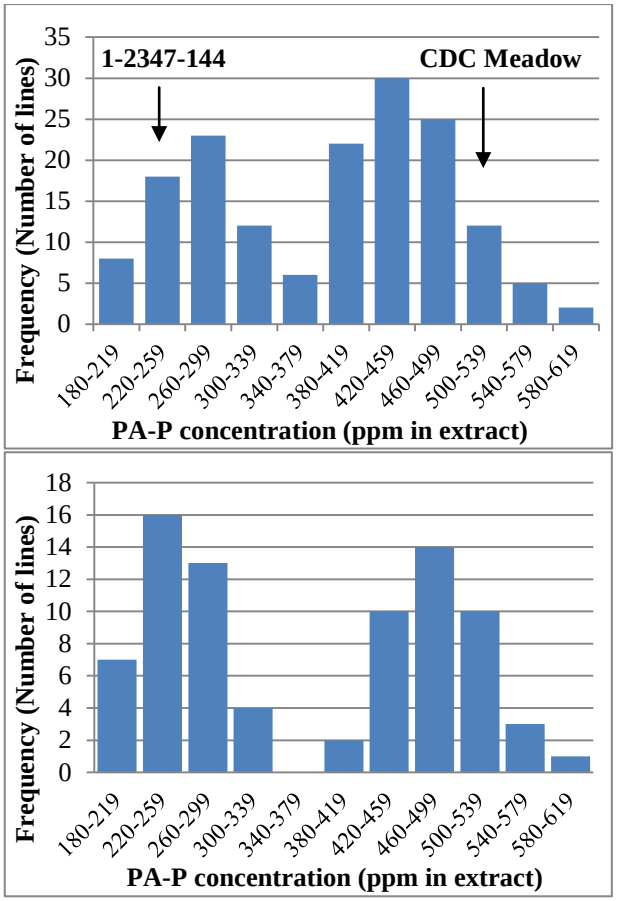

Figure 5.1. Distribution of PA-P concentration for RILs of PR-15 (n=163, graph A, top) and the subset RILs of PR-15 (n=80, graph B, bottom), 2012 Rosthern.

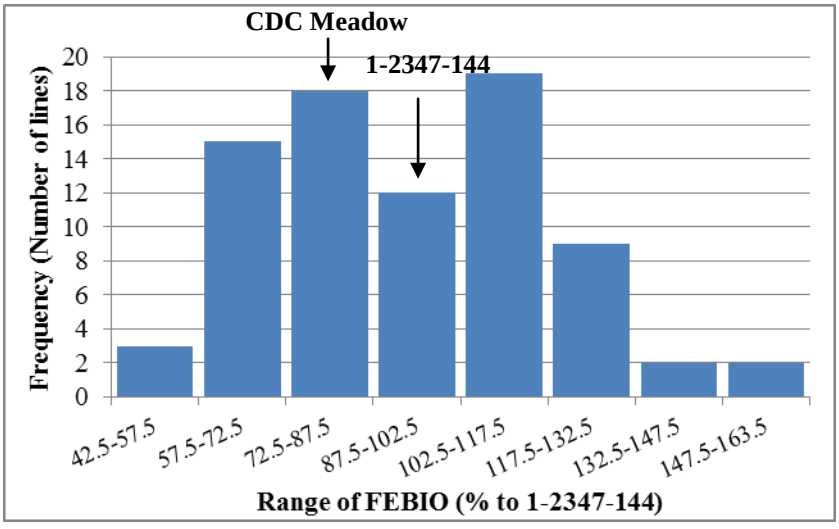

Figure 5.2. Frequency distribution of mean FEBIO of two replications (estimated by the percentage relative to low phytate parent, 1-2347-144) for the subset of 80 PR-15 RILs, 2012 Rosthern.

5.3.3 Correlation of FECON, FEBIO and PA-P

From the Pearson correlation test, the correlation coefficient between FECON and FEBIO and between FECON and PA-P were 0.05 and -0.02 (both were not significant with P-values >0.05), respectively. Samples were grouped into 2 levels according to the phytate standard (40 lines grouped as high PA, 40 lines grouped as low PA, also including the low PA parent 1-2347-144 and the normal PA parent CDC Meadow). Figure 5.3 presents the scatterplot and the tendency of the correlation between the PA-P and FEBIO in 2012 Rosthern. The correlation between the PA-P and FEBIO was found to be significantly negative (P-values <0.01). PR-15 RILs with lower PA-P concentration tended to have higher FEBIO. The Pearson correlation coefficient between PA-P and FEBIO was -0.83, which indicated that the two traits were highly correlated.

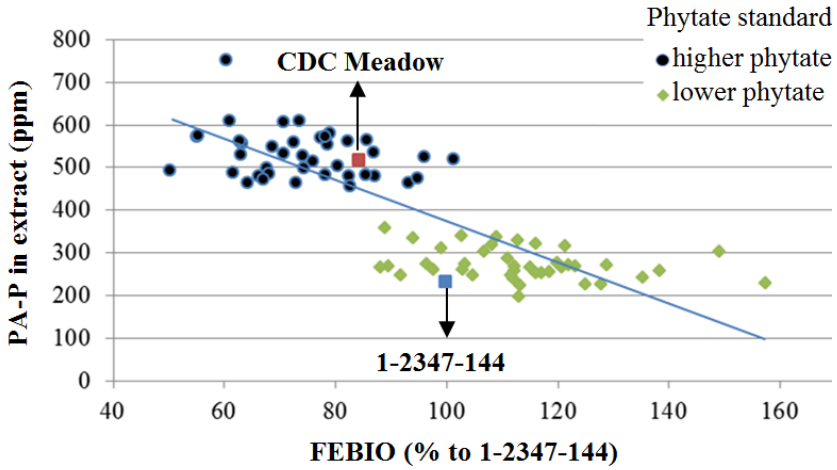

Figure 5.3. Scatterplot between the PA-P (ppm in extract) and FEBIO (% to 1-2347-144) of the subset RILs (n=80), with two replications in 2012 Rosthern. Blue square - the low phytate parent (1-2347-144) and red square - the normal phytate parent (CDC Meadow).

5.4 Discussion and Conclusion

In recent years, biofortified crops have been developed to reduce nutritional deficiencies (Campion et al., 2009; Larson et al., 1998; Larson et al., 2000; Raboy et al., 2000; Warkentin et al., 2012; Wilcox et al., 2000). It is necessary to test for the consistent nutritional benefit of the biofortified crop. Nutritional benefits are often quantitative traits associated with many genes. In contrast to agronomic traits such as lodging and plant height, the nutritional status in the seeds cannot be easily measured visually. Identification of nutritional benefits which are consistent and heritable would be beneficial.

The FEBIO is a quantitative trait which is influenced by many factors such as iron type (non-heme or heme iron), its promoters (such as AA) and its inhibitors (such as phytate and

polyphenols) (Anderson and McLaren, 2012; Jin et al., 2009). Increasing the amount of promoters, decreasing the amount of inhibitors or increasing the accumulation of high-bioavailable iron type could increase FEBIO in staple foods. Due to its complicated mechanism, not many studies have been directly aim at FEBIO in crops.

Decreasing phytate concentration in a food matrix can significantly increase the absorption of non-heme iron (Anderson and McLaren, 2012; Bañuelos et al., 2008; Kalgaonkar et al., 2008). However, previous reports have shown disagreement about whether or not there is a correlation between phytate concentration and FEBIO in crops. Phytate concentration was found not being correlated with FEBIO in 24 bean varieties and 15 rice varieties, respectively (Glahn et al., 2002b; Welch et al., 2000). However, Aluru et al. (2011) reported that phytate concentration was negatively correlated with FEBIO in transgenic maize. Since the bean and rice studies used genotypes with different traits, there might have been other factors such as polyphenols which affected the correlation between phytate concentration and FEBIO. Meanwhile, the molar ratio of PA:Fe of 15 rice varieties were all larger than 13:1, and phytate concentration had limited effect on FEBIO at that molar ratio (Glahn et al., 2002a).

Increasing non-heme iron type with relatively higher bioavailability can also increase iron absorption. Aluru et al. (2011) and Drakakaki et al. (2005) indicated that using a transgenic approach to introduce a soybean ferritin gene can increase both FECON and FEBIO, and they are correlated. However, by studying the crop varieties in the market, iron concentration was not significantly correlated with FEBIO (Glahn et al., 2002b, Lung'aho et al., 2011; Oikeh et al., 2004; Vasconcelos et al., 2003; Welch et al., 2000).

Quantitative trait locus (QTL) analysis was a good tool to study the inheritance of FEBIO. Lung'aho et al. (2011) reported ten QTLs for FEBIO in maize which explained 54 % of the variance. Šimić et al. (2012) reported three QTLs for ratios (Fe:P, Zn:P and Mg/P) that were related to mineral bioavailability, these QTLs were found close to the phytase gene in maize.

In the PR-15 RILs arising from a cross between low phytate line (1-2347-144) and normal phytate variety (CDC Meadow), the FEBIO was highly negatively correlated with PA-P. The frequency distribution showed that FEBIO followed a bimodal pattern, thus, the FEBIO trait appears to be controlled primarily by a single major gene in PR-15 RILs. Previously, the low phytate trait in pea was shown to be controlled by a single recessive gene (Rehman et al.,

2012). Since the FEBIO in PR-15 is highly correlated with PA-P, the FEBIO in the PR-15 RILs might also be mainly controlled by pleiotropic effects of the same gene following Mendelian inheritance.

In this study, although the FEBIO was highly correlated with PA-P, the scatter plot separated into two clusters due to the gap of PA-P in the subset (Figure 5.3), meanwhile, the bimodal segregation of FEBIO was not as perfect as the bimodal segregation of PA-P in PR-15 RILs (Figure 5.2). The two parents (CDC Meadow and 1-2347-144) were found to be relatively close in FEBIO, but still to differ significantly. Additionally, there were four lines had more than 132 % FEBIO compared with the low phytate parent (1-2347-144), thus showing transgressive segregation. These results indicate that the parent, CDC Meadow, might also have minor genes that enhanced FEBIO in the offspring. Further QTL study may aid in detecting those genes. PR-15 was developed to study the inheritance of phytate, and the selection of parents was based on phytate concentration. The inheritance of FEBIO was considered as a beneficial trait to study in PR-15 RILs. However, in the Phytate Study and Seed Coat Study, differences in FEBIO between low phytate lines and CDC Meadow were smaller than between the low phytate lines and the other normal phytate varieties, and this difference became much smaller in the Rosthern location, where PR-15 RILs were grown in this experiment.

In this case, there must be some unknown factors in CDC Meadow to affect FEBIO, and the PR-15 population is not a perfect model to study FEBIO. Future research could include determination of whether the major gene that controls FEBIO in PR-15 RILs is the same gene that controls PA-P, conducting more trials in different locations and more years to minimize the environmental effect, generating RILs with two parents having greater difference in FEBIO, and QTL analysis of FEBIO with iron concentration as a co-factor to potentially identify minor genes affecting FEBIO in PR-15 lines.

6.0 Experiment 4 (Chicken study):

The effects of low phytate pea on body weight and hemoglobin concentration of chicken

6.1 Introduction

Iron is one of the essential nutrients for humans and animals. The low phytate field pea lines (1-150-81 and 1-2347-144) were shown to have higher inorganic phosphorus concentration than normal phytate varieties (Warkentin et al., 2012). The lowering of phytate concentration in food can increase the bioavailability of iron (Glahn et al., 2002a). In Experiment 1 of this thesis, the *in vitro* digestion/Caco-2 cell culture bioassay showed that the two low phytate pea lines had higher FEBIO than normal phytate varieties.

In this experiment, an *in vivo* study was used with the objective of confirming the results of the *in vitro* study. Three chicken trials were conducted to examine and compare the chicken body weight (BW) and hemoglobin concentration (Hb) between the treatments using the low phytate pea lines and normal phytate varieties. Chicken Study 1, coded as CTR 1201, was conducted from March 28th, 2012 for a period of 35 days. CTR 1216, Chicken Study 2, was started on October 25th, 2012 for the period of 24 days, with the objective of idealizing iron level in the diets. The last chicken trial, Chicken Study 3 (CTR 1304), was started on March 05th, 2013 for a period of 35 days.

In August 2012, the ground pea samples and feeds from Chicken Study 1 were evaluated for IN-P, PA-P and FECON using the methods described in sections 3.2.2 to 3.2.4. In May 2013, the ground pea samples and the feeds from all three chicken studies were evaluated for FECON using Inductively Coupled Plasma (ICP) in the laboratory of Dr. Raymond Glahn (USDA-ARS, Ithaca, NY, USA). In August 2013, several ingredients used in the diets were tested for FECON by AAS method (section 3.2.4).

6.2 Chicken study 1 (CTR 1201)

6.2.1 Materials and Methods

Plant materials:

Two low phytate pea lines (1-150-81 and 1-2347-144) and two normal phytate field pea varieties (CDC Bronco and CDC Meadow) were used as ingredients in the diets. These four pea varieties were all from the 2010 harvest at the University of Saskatchewan Kernen Farm. Peas were ground with a Jacobson Full Circle Hammer mill using a 3.2 mm screen before

adding into the feed.

Experimental methods:

Chicken Study 1 had 4 treatments with 6-replications with 4 birds (Ross x Ross 308 male chicks) per replication (total 24 birds * 4 treatments = 96 birds). The experimental design was RCBD. Treatments were started when the chickens were grown to day 7. Before that, all birds were fed with a commercial diet to balance the nutrition intake and to maintain health. Four birds were placed in each battery cage (50 cm width × 85 cm length × 25 cm height) at a room temperature of 31°C (started day 0), 21°C (started day 24) and lighting profile of light:dark=20:4 (light intensity = 20 lux). Birds were moved to bigger grow out batteries (50 cm width × 70 cm length × 45 cm height) after day 21. Subsequently, the mortality was recorded.

For each week, the BW of each chicken and the feed intake of each battery were recorded. At the final day of the experiment, the chicken blood samples were collected before the eight-hour fasting of the birds. The blood samples were later used for the hematocrit (Hct) evaluation and Hb assay. The Hct followed the microhematocrit method, and Hb assay followed the method described in the paper of Tako et al. (2010) using the cyanmethemoglobin method. The treatment information and the feed ingredients for Chicken Study 1 are presented in Table 6.1.

The ANOVA tests of the chicken BW, Hb and Hct were evaluated following Proc Mixed in SAS ® V9.3 (SAS Institute Inc. 2011, NC, USA) to evaluate the factor of treatment. In this analysis, treatment was considered as a fixed effect and replication was considered as a random effect. When a significant difference was determined by ANOVA, means for variety, environment or variety*environment were separated using Tukey's Mean Comparison with a significance level of $P \leq 0.05$. Finally, these results were displayed in figures using MS Excel (Microsoft Canada Inc., Mississauga, ON, Canada).

Table 6.1. Chicken Study 1 (CTR 1201), treatment information and the feed ingredients.

Diet ingredients	%
Pea[1]	87.01
Canola Oil	7.20
Common Salt (NaCl)	0.44
Dicalcium-Phosphate	0.67
Limestone	2.10
Vitamin/Mineral Premix (Fe free)[2]	0.50
Choline Chloride	0.10
Celite	1.50
DL Methionine	0.48

[1] L-150, treatment with diet containing low phytate line 1-150-81. L-2347, treatment with diet containing low phytate line 1-2347-144. N-Bronco, treatment with diet containing normal phytate variety CDC Bronco. N-Meadow, treatment with diet containing normal phytate variety CDC Meadow.

[2] Supplied per kilogram of diet: vitamin A (retinyl acetate + retinyl palmitate), 11000 IU ; vitamin D, 2200 IU; vitamin E (dl-α-topheryl acetate), 30 IU; menadione, 2mg; thiamine, 1.5 mg; riboflavin, 6 mg; niacin, 60 mg; pyridoxine, 4 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 10 mg; folic acid, 0.6 mg; biotin, 0.15 mg; iron, 0.0 mg; zinc, 80 mg; manganese, 80 mg; copper, 10 mg; iodine, 0.8 mg; selenium, 0.3 mg; calcium carbonate, 500 mg.

6.2.2 Experimental results

Chicken BW at the age of day 7, day 14, day 21, day 28 and day 35 had no significant difference among the four treatments (Table 6.2). Chicken Hct and Hb of Chicken Study 1 at the age of day 35, shown in Table 6.3, indicated that the four treatments had no significantly effect on Hct and Hb. In this chicken trial, totally nine birds died during the experimental period, and at the end of the experiment five birds were found to have ascites. Since there were in total 96 birds in this experiment, this mortality rate is relatively high compared with previous experience.

Similar to the results of Experiment 1, the low phytate lines (1-150-81 and 1-2347-144) contained higher IN-P and lower PA-P, than the normal phytate varieties, and a similar tendency was detected in the feeds (Table 6.4). However, the FECON of the feed was approximately three to four times greater compared with the ground pea alone. However, as mentioned in the introduction, the FECON for Chicken Study 1 was first tested by AAS but was not considered as a problem at that time, until it was reconfirmed by ICP later. Note that this FECON data from ICP were collected after all three chicken studies had been completed.

Table 6.2. ANOVA table of BW (g) for four treatments (L-150, L-2347, N-Bronco and N-Meadow) from the age from day 7 to day 35.

Effect	Num DF	F-values				
		D7 BW	D14 BW	D21 BW	D28 BW	D35 BW
Treatment	3	1.41 ^{ns}	0.81 ^{ns}	2.31 ^{ns}	1.30 ^{ns}	0.60 ^{ns}

Notes: L-150, treatment with diet containing low phytate line 1-150-81. L-2347, treatment with diet containing low phytate line 1-2347-144. N-Bronco, treatment with diet containing normal phytate variety CDC Bronco. N-Meadow, treatment with diet containing normal phytate variety CDC Meadow. ns, no significant $p>0.05$. Num DF, degrees of freedom.

Table 6.3. ANOVA table of Hb (g/dl) and Hct (%) for four treatments (L-150, L-2347, N-Bronco and N-Meadow) at the age of day 35.

Effect	Num DF	F-values	
		Hb	Hct
Treatment	3	1.15 ^{ns}	0.63 ^{ns}

Notes: L-150, treatment with diet containing low phytate line 1-150-81. L-2347, treatment with diet containing low phytate line 1-2347-144. N-Bronco, treatment with diet containing normal phytate variety CDC Bronco. N-Meadow, treatment with diet containing normal phytate variety CDC Meadow. ns, no significant $p>0.05$. Num DF, degrees of freedom.

Table 6.4. Chicken Study 1, IN-P (ppm), PA-P (ppm) and FECON (ppm) of the four ground peas (1-150-81, 1-2347-144, CDC Bronco and CDC Meadow) and four treatments (L-150, L-2347, N-Bronco and N-Meadow).

		IN-P		PA-P		FECON[2]	
Variety	Type[1]	Mean	SEM	Mean	SEM	Mean	SEM
1-150-81	ground pea	1615	9	2007	160	48.0	0.4
	diet L-150	2141	70	827	24	159.7	15.7
1-2347-144	ground pea	1457	43	1900	125	47.7	1.2
	diet L-2347	1876	49	1117	140	136.0	8.1
CDC Bronco	ground pea	429	30	3253	89	46.2	1.2
	diet N-Bronco	1574	53	2428	274	132.8	14.5
CDC Meadow	ground pea	488	21	3235	178	45.9	1.1
	diet N-Meadow	1442	53	1918	164	127.3	8.3

[1] L-150, treatment with diet containing low phytate line 1-150-81; L-2347, treatment with diet containing low phytate line 1-2347-144; N-Bronco, treatment with diet containing normal phytate variety CDC Bronco; N-Meadow, treatment with diet containing normal phytate variety CDC Meadow. SEM, standard error of the mean.
[2] FECON results are from ICP.

6.3 Chicken study 2 (CTR 1216)

Since the low phytate lines and normal phytate pea varieties did not differ in their effects on chicken performance in Chicken Study 1, Chicken Study 2 was conducted to attempt to refine the iron concentrations in the diets. Since the FECON results from AAS were not considered as a problem when designing Chicken Study 2, and based on the mortality rate in Chicken Study 1, a hypothesis arose that the iron concentration in the diets in Chicken Study 1 might have been too low to meet the minimum need of the birds. Therefore, the second chicken study was conducted to idealize the iron concentration in the feed.

6.3.1 Materials and Methods

Plant materials:

One of the low phytate lines (1-2347-144) and its parent CDC Bronco (normal phytate variety) were used as the feed ingredients in this study. Both were from the 2010 harvest at the Kernen Farm. Peas were ground with a Jacobson Full Circle Hammermill (< 3.2mm screen) before feed mixing.

Experimental methods:

Table 6.5 shows details of the ingredients of the factorial experiment. The iron supplement was ferric citrate (Tako et al., 2010). Four levels of iron were used in this experiment: 1) basal diet with no added iron; 2) basal diet with additional 12 mg Fe/kg feed; 3) basal diet with additional 24 mg Fe/kg feed; 4) basal diet with additional 36 mg Fe/kg feed.

A factorial RCBD was used for the two pea sources with 4 different levels of iron supplement, which made the total number of treatment 8 (2 varieties *4 levels of iron). The basic environment for the birds, such as lighting and temperature, were maintained in the same way as in Chicken Study 1. Each treatment was replicated three times, with four birds per replication. The total trial duration was 24 days. The treatments started on day 0. The mortality was recorded daily throughout the experiment. BW, Hct and Hb were measured every 6 days followed the same methods described in Chicken Study 1.

The ANOVA tests of the chicken BW, Hb and Hct were evaluated following Proc Mixed in SAS ® V9.3 (SAS Institute Inc. 2011, NC, USA) to evaluate the factors of treatment, variety and their interaction. In this analysis, treatment and variety was considered as a fixed effect and replication was considered as a random effect. When a significant difference was determined by ANOVA, means for variety, environment or variety*environment were

separated using Tukey's Mean Comparison with a significance level of $P \leq 0.05$. Finally, these results were displayed in figures using MS Excel software (Microsoft Canada Inc., Mississauga, ON, Canada).

Table 6.5. Chicken Study 2 (CTR 1216), treatment information and the feed ingredients.

	Treatment[1]			
Diet ingredients (%)	(1),(5)	(2),(6)	(3),(7)	(4),(8)
Pea	85.55	85.55	85.55	85.55
Canola Oil	5.08	5.08	5.08	5.08
Common Salt (NaCl)	0.37	0.37	0.37	0.37
Dicalcium-Phosphate	1.37	1.37	1.37	1.37
Limestone (%)	1.67	1.67	1.67	1.67
Vitamin/Mineral Premix (Fe free)[2]	0.50	0.50	0.50	0.50
Choline Chloride	0.10	0.10	0.10	0.10
L-threonine	0.31	0.31	0.31	0.31
L-Tryptophan	0.07	0.07	0.07	0.07
Casein	3.38	3.38	3.38	3.38
DL Methionine	0.60	0.60	0.60	0.60
Ferric citrate (g/kg feed)	0.0000	0.0685	0.1370	0.2055

[1] (1), treatment with basal diet containing 1-2347-144 (44 mg Fe/kg); (2), treatment with basal diet containing 1-2347-144 and extra 12 mg Fe/kg feed; (3), treatment with basal diet containing 1-2347-144 and extra 24 mg Fe/kg feed; (4), treatment with basal diet containing 1-2347-144 and extra 36 mg Fe/kg feed; (5), treatment with basal diet containing CDC Bronco (44 mg Fe/kg); (6), treatment with basal diet containing CDC Bronco and extra 12 mg Fe/kg feed; (7), treatment with basal diet containing CDC Bronco and extra 24 mg Fe/kg feed; (8), treatment with basal diet containing CDC Bronco and extra 36 mg Fe/kg feed.
[2] Supplied per kilogram of diet: vitamin A (retinyl acetate + retinyl palmitate), 11000 IU ; vitamin D, 2200 IU; vitamin E (dl-α-topheryl acetate), 30 IU; menadione, 2mg; thiamine, 1.5 mg; riboflavin, 6 mg; niacin, 60 mg; pyridoxine, 4 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 10 mg; folic acid, 0.6 mg; biotin, 0.15 mg; iron, 0.0 mg; zinc, 80 mg; manganese, 80 mg; copper, 10 mg; iodine, 0.8 mg; selenium, 0.3 mg; calcium carbonate, 500 mg.

6.3.2 Experimental results

The P-values from ANOVA test of Hb, Hct and BW on the birds at day 6, 12, 18, 24 are summarized in Table 6.6. Nearly all the P-values in this table were greater than 0.05, which indicates the treatments did not affect the Hb, Bct and BW in this experiment.

The diets contained more IN-P and lower PA-P than the ground pea (Table 6.7). The low phytate diets contained similar IN-P compared to the normal phytate diets, but two to three times lower PA-P than the normal phytate diets. FECON in the diets was three to five times higher than that in the ground peas. Same as Chicken Study 1, the FECON in the diets were four to six times higher than in the ground pea samples. As more iron supplement was added,

more FECON was obtained (diet 2, 3, 4 and 6, 7, 8). Ten birds died during the experimental period; since there were total 96 birds in this experiment, this mortality is still relatively high compared with previous experience.

Table 6.6. ANOVA table of Hb (g/dl), Hct (%) and BW (g) of the 8 treatments across the experimental period (day 6, day 12, day 18 and day 24).

	F-values			
Effect	Day 6	Day 12	Day 18	Day 24
variety (Hb)	0.86 ns	0.05 *	0.42 ns	0.83 ns
trt (Hb)	0.02 ns	0.88 ns	0.67 ns	0.28 ns
variety*trt (Hb)	0.87 ns	0.80 ns	0.55 ns	0.45 ns
variety (Hct)	0.46 ns	0.09 ns	0.02 *	0.19 ns
trt (Hct)	0.24 ns	0.57 ns	0.08 ns	0.06 ns
variety*trt (Hct)	0.26 ns	0.90 ns	0.60 ns	0.46 ns
variety (BW)	0.56 ns	0.56 ns	0.38 ns	0.34 ns
trt (BW)	0.13 ns	0.17 ns	0.28 ns	0.14 ns
variety*trt (BW)	0.33 ns	0.06 ns	0.18 ns	0.07 ns

Notes: ns, no significant $p>0.05$; *, significant at $p\leq0.05$.

Table 6.7. Chicken Study 2, IN-P (ppm), PA-P (ppm) and FECON (ppm) of the two ground pea (1-2347-144 and CDC Bronco) and the eight treatment diets.

		IN-P		PA-P		FECON[2]	
Variety	Type[1]	Mean	SEM	Mean	SEM	Mean	SEM
1-2347-144	ground pea	1834	133	1855	204	43.0	0.6
(low phytate)	diet (1)	3080	213	702	12	239.1	5.3
	diet (2)	3478	117	494	83	180.8	41.5
	diet (3)	3105	275	491	72	197.0	30.2
	diet (4)	3807	86	899	34	243.6	29.4
CDC Bronco	ground pea	439	22	3486	68	46.4	1.1
(normal	diet (5)	3242	187	2126	76	203.6	27.4
phytate)	diet (6)	2247	310	1447	68	202.4	11.4
	diet (7)	2560	439	1175	68	230.4	13.5
	diet (8)	2115	425	903	68	257.6	31.8

[1] (1), treatment with basal diet containing 1-2347-144 (44 mg Fe/kg); (2), treatment with basal diet containing 1-2347-144 and extra 12 mg Fe/kg feed; (3), treatment with basal diet containing 1-2347-144 and extra 24 mg Fe/kg feed; (4), treatment with basal diet containing 1-2347-144 and extra 36 mg Fe/kg feed; (5), treatment with basal diet containing CDC Bronco (44 mg Fe/kg); (6), treatment with basal diet containing CDC Bronco and extra 12 mg Fe/kg feed; (7), treatment with basal diet containing CDC Bronco and extra 24 mg Fe/kg feed; (8), treatment with basal diet containing CDC Bronco and extra 36 mg Fe/kg feed. SEM, standard error of the mean.

[2]FECON results are from ICP.

6.4 Chicken study 3 (CTR 1304)

The first two chicken studies used diets containing approximately 87 % peas, as this methodology had been successfully utilized before (Ebsim, 2013). The third chicken study used diets containing 40 % peas following a protocol similar to the one used successfully by Tako and Glahn (2011).

6.4.1 Materials and Methods

Plant materials:

Two low phytate lines (1-150-81 and 1-2347-144) and two normal phytate varieties (CDC Bronco and CDC Meadow) were ground using a Jacobson Full Circle Hammermill, screen <3.2mm. All peas were derived from the 2012 harvest at the Kernen Farm, Saskatoon.

Experimental methods:

Table 6.8. Chicken Study 3 (CTR 1304), treatment information and the feed ingredients.

	Treatment[1]	
Diet ingredients (%)	L-150, L-2347, N-Bronco, N-Meadow	Control
Pea	40.00	40.00
Corn	43.98	43.98
Casein	10.00	10.00
Canola oil	1.87	1.87
Limestone	1.35	1.35
Dicalcium-Phosphate	0.99	0.99
Sodium Chloride	0.36	0.36
Vitamin/Mineral Premix (Fe free)[2]	0.50	0.50
DL Methionine	0.54	0.54
L Threonine	0.26	0.26
Choline chloride	0.10	0.10
Ferric Citrate	0.01	0.06
L-Tryptophan	0.04	0.04

[1] L-150, treatment with diet containing low phytate line 1-150-81 and extra 0.01 % ferric citrate; L-2347, treatment with diet containing low phytate line 1-2347-144 and extra 0.01 % ferric citrate; N-Bronco, treatment with diet containing normal phytate variety CDC Bronco and extra 0.01 % ferric citrate; N-Meadow, treatment with diet containing normal phytate variety CDC Meadow and extra 0.01 % ferric citrate.

[2] Supplied per kilogram of diet: vitamin A (retinyl acetate + retinyl palmitate), 11000 IU ; vitamin D, 2200 IU; vitamin E (dl-α-topheryl acetate), 30 IU; menadione, 2mg; thiamine, 1.5 mg; riboflavin, 6 mg; niacin, 60 mg; pyridoxine, 4 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 10 mg; folic acid, 0.6 mg; biotin, 0.15 mg; iron, 0.0 mg; zinc, 80 mg; manganese, 80 mg; copper, 10 mg; iodine, 0.8 mg; selenium, 0.3 mg; calcium carbonate, 500 mg.

A RCBD was used in this study with five treatments (Table 6.8). Considering the results from Chicken Study 2, all diets for each treatment contained 0.01 % of ferric citrate supplement, except the control diet (CDC Bronco) which had 0.06 % added ferric citrate. The basic environment for the birds, including lighting and temperature, were the same as used in Chicken Study 1. Each treatment was replicated six times with four birds / replication. The total trial duration was 35 days. The treatment started on the age of day 0 of the birds. The mortality was recorded daily throughout the experiment. BW and Hb (not Hct) were measured every 7 days following the same methods described in Chicken Study 1.

The ANOVA tests of the chicken BW and were evaluated following Proc Mixed in SAS ® V9.3 (SAS Institute Inc. 2011, NC, USA) to evaluate the factor of treatment. In this analysis, treatment and variety was considered as a fixed effect and replication was considered as a random effect. When a significant difference was determined by ANOVA, means for variety, environment or variety*environment were separated using Tukey's Mean Comparison with a significance level of $P \leq 0.05$. Finally, these results were displayed in figures using MS Excel software (Microsoft Canada Inc., Mississauga, ON, Canada).

6.4.2 Experimental results

The P-values from ANOVA table of BW on day 7, 14, 21 showed there were no significant differences among treatments (Figure 6.1). However, on day 28 and 35, P-values of BW indicated a significant effect of treatment on BW; the BW of the birds fed with CDC Meadow (N-Meadow) was the lowest. The P-values of the hemoglobin (g/dl) on each day of measurement showed that the treatments had no significant effect. On day 28, the Hb of treatment L-150 was less than N-Bronco and N-Meadow; similarly, birds fed diets containing 1-2347-144 had lower Hb value than those fed diets containing CDC Bronco. On the final day (D35), the Hb from the control treatment was significantly less than that from treatment N-Bronco.

IN-P was higher and PA-P was lower in the diets than the ground pea. The PA-P of the normal phytate treatments (N-Bronco and N- Meadow) was four to five times higher compared with the PA-P of the low phytate treatments (Table 6.9). The FECON of the diet was four times higher than FECON of the ground sample. Furthermore, one of the low phytate lines (1-2347-144) contained higher FECON in both ground pea and diet than the other three pea varieties (Table 6.9). The FECON in the other four treatments (L-150, N-Bronco, N-Meadow and Control) did not differ significantly.

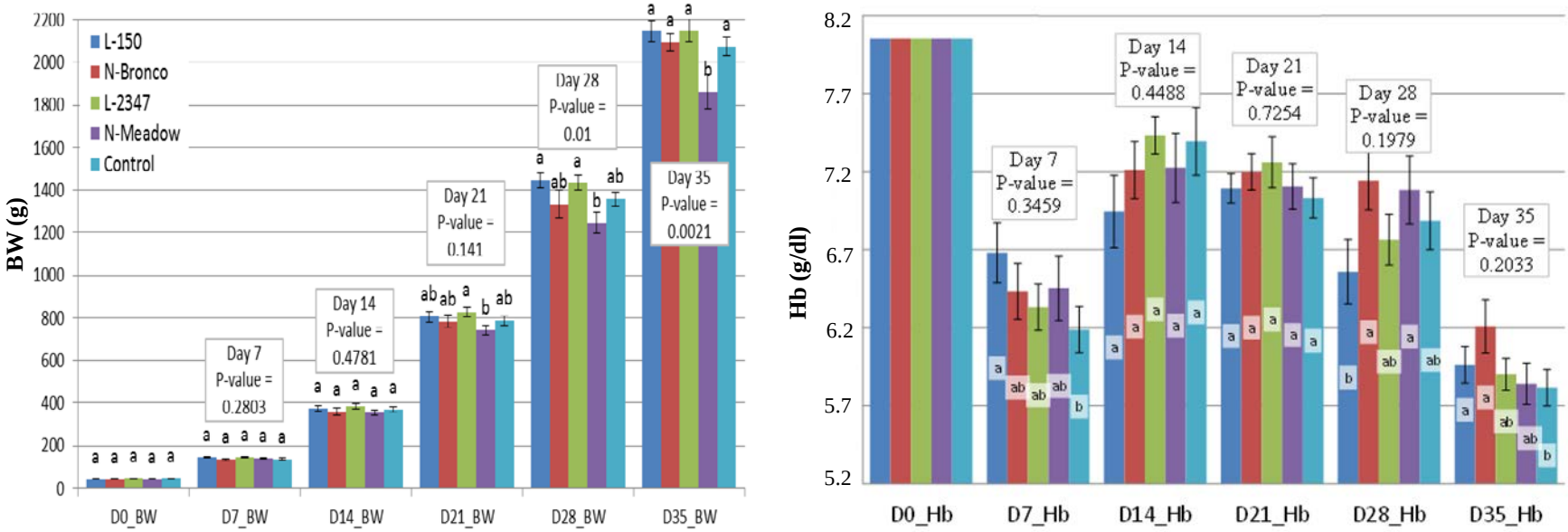

Figure 6.1. Chicken Study 3, body weight (BW, g) and hemoglobin (Hb, g/dl) of the day 0, 7 14, 21, 28 and 35 of the bird age. L-150, treatment with diet containing low phytate line 1-150-81 and extra 0.01 % ferric citrate; L-2347, treatment with diet containing low phytate line 1-2347-144 and extra 0.01 % ferric citrate; N-Bronco, treatment with diet containing normal phytate variety CDC Bronco and extra 0.01 % ferric citrate; N-Meadow, treatment with diet containing normal phytate variety CDC Meadow and extra 0.01 % ferric citrate. Letters above/within bars indicates significant differences (Tukey's Mean Comparison, P <0.05). Error bar shows the standard error of the mean.

Seven birds died during this experiment. At the final day of the experiment, two birds were found having ascites. The total number of birds in this experiment was 120. The mortality rate was lower than in the first two chicken studies.

Since the higher FECON detected in diets compared to the ground pea samples was reconfirmed by ICP and this pattern presented consistently through all three chicken studies, it was found that some ingredients in the diets were a source of iron and provided dietary Fe beyond the research expectation. Ingredients, which were suspected to be impure, were tested by AAS in August 2013. Four ingredients were chosen, that is, L-threonine, limestone, dicalcium-phosphate and sodium chloride. The results are shown in Table 6.10.

Table 6.9. Chicken Study 3, IN-P (ppm), PA-P (ppm) and FECON (ppm) of the four ground pea (1-150-81, 1-2347-144, CDC Bronco and CDC Meadow) and five treatments (L-150, L-2347, N-Bronco, N-Meadow and Control).

		IN-P		PA-P		FECON	
Variety	Type	Mean	SEM	Mean	SEM	Mean	SEM
1-150-81	ground pea	2464	269	1651	0	51.6	0.8
	diet L-150	2996	144	767	340	199.7	22.6
1-2347-144	ground pea	2748	148	1719	340	90.0	1.3
	diet L-2347	3092	224	722	112	244.1	67.4
CDC Bronco	ground pea	471	63	3418	408	45.8	0.6
	diet N-Bronco	2771	195	2942	340	182.7	16.4
CDC Meadow	ground pea	539	55	2670	68	45.4	0.8
	diet N-Meadow	3079	238	2330	408	196.0	4.5
Control	diet Control	2379	191	2942	68	195.7	22.6

Notes: L-150, treatment with diet containing low phytate line 1-150-81 and extra 0.01 % ferric citrate; L-2347, treatment with diet containing low phytate line 1-2347-144 and extra 0.01 % ferric citrate; N-Bronco, treatment with diet containing normal phytate variety CDC Bronco and extra 0.01 % ferric citrate; N-Meadow, treatment with diet containing normal phytate variety CDC Meadow and extra 0.01 % ferric citrate. FECON results are from ICP. SEM, standard error of the mean.

Table 6.10. AAS test for iron concentration (ppm) of the selected ingredients used in the three chicken studies, and the calculated iron concentration that these ingredients contributed to the diets.

			Chicken study 1		Chicken study 2		Chicken study 3	
Ingredient	Fe (ppm)	SD	% in diet	Fe contributed to diet (ppm)	% in diet	Fe contributed to diet (ppm)	% in diet	Fe contributed to diet (ppm)
L-threonine	2.5	0	0.48	0.01	0.06	0.001	0.26	0.006
limestone	483	51	2.10	10.14	1.67	8.07	1.35	6.52
dicalcium-phosphate	10798	2553	0.67	72.35	1.37	147.93	0.99	106.90
sodium chloride	16.8	1	0.44	0.07	0.37	0.06	0.36	0.06

Notes: SD, Standard Deviation

6.5 Discussion

Low-phytate lines from different crops had been evaluated in non-ruminant animals to test phosphorus availability. Low phytate maize had approximately 50 % more available phosphorus than normal phytate maize (Spencer et al., 2000; Sugiura et al., 1999). Fish fed with low phytate barley had 50 % lower fecal phosphorus content, which indicates more available phosphorus in the diet (Sugiura et al., 1999).

The methodology of the chicken studies conducted in this thesis was adopted in part from the *in vivo* chicken study published by Tako et al. (2010). They used soybean meal, corn oil, corn starch, choline chloride, DL-Methionine, and non-iron vitamin-mineral premix to compose the experimental diet (Tako et al., 2010). These diets are deficient in Na, Ca and P and therefore salt, limestone and dicalcium phosphate were added to the diets in this research.

After the first chicken study, the FECON test was conducted on chicken diets by AAS immediately, which was later confirmed by the result from an ICP test in May 2013. From the AAS test, the large difference between the FECON in diets and ground pea was firstly considered as a result of improper reading of AAS of the prepared diet. However, ICP confirmed the previous result from AAS that the diet actually contained much more iron than expected, so that all diets provided more than enough iron for normal chicken growth.

Thus, AAS was conducted to test if the ingredients (i.e. dicalcium-phosphate, limestone, L-threonine) used to prepare the diets in the three chicken studies were impure and if they contained extra iron. The data are summarized in Table 6.10. Since the iron in diets was designed to arise only from the pea samples and was controlled and calculated based on the iron concentration in the peas, the extreme higher iron concentration in the feed might have come from the other ingredients. Table 6.10 shows that the limestone and dicalcium-phosphate ingredients used contained extremely high iron concentration. The iron contributed to the diets by these ingredients was far greater than that contributed by the peas, and this explains the higher iron concentration in the diets than expected, and the lack of response to the pea

treatments differing in PA-P.

6.6 Conclusion

Overall among the three chicken trials, the diets differing in low phytate and normal phytate pea ingredients had no significant effect on the chicken BW and Hb. From the result of the iron concentration testing of diets and ingredients, the experiment did not exclude the impure ingredients which provided extra iron to the chickens and overwhelmed the potential effects of the diets. Since the birds in each treatment had enough iron for growth, the body weight and hemoglobin level were not influenced by the differences in available iron from the low and normal phytate treatments. These chicken studies emphasized that when testing nutritional availability of trace minerals like iron, the diets and all experimental materials should be carefully chosen to exclude the nutrition from the unexpected sources and to insure the animals only have access to the controllable experimental factors.

7.0 General discussion, conclusion and further research

7.1 General discussion

Iron bioavailability of food is influenced by its total iron concentration and promoters (such as meat, fish and ascorbic acid) and anti-nutritional factors (such as phytate and polyphenols). Differing from some phenotypic traits (such as plant height and yield), breeding that focuses on nutritional traits requires additional laboratory work. For traits associated with nutrition bioavailability, it is not a simple measurement of the amount of nutrition in crops; instead, it requires additional *in vivo* human/animal experiments or adequate *in vitro* experiments.

Low phytate is one of the biofortification techniques to increase iron bioavailability. Phytate is a strong chelator that can inhibit the availability of nutrients including phosphorus, iron and zinc. Low phytate mutants were developed in several crops which firstly targeted the enhancement of phosphorus utilization especially to the farm non-ruminant animals, and protecting the environment from eutrophication (Campion et al., 2009; Shi et al., 2003; Thavarajah et al., 2013; Wilcox et al., 2000). These low phytate lines typically had 40 to 90 % of reduction of phytate-phosphorus concentration compared to the normal phytate varieties from which they were derived, and had increased phosphorus availability. Other nutritional benefits of biofortified low phytate lines were also studied. Low phytate rice not only increased inorganic phosphorus concentration, but also increased the availability of Fe, Zn and Ca (Ren et al., 2007). The common bean low phytate line contained more soluble Fe than normal phytate common bean (Campion et al., 2009).

Several low phytate crop studies also showed an increase of iron bioavailability, but the correlation between iron bioavailability and phytate concentration has not been completely consistent. The low phytate maize line (*lpa1-1*) was developed with 60 % lower phytate concentration and 1.5 times higher iron bioavailability than normal phytate variety A188 (Aluru et al., 2011). They also studied transgenic low phytate maize lines and found a

significant negative correlation between iron bioavailability and phytate concentration. Inserting a heat-tolerant phytase gene in maize also resulted in a significant increase of iron bioavailability (Drakakaki et al., 2005). In Experiment 1 Phytate Study, a 50 % lower phytate concentration resulted in a 1.5 to 2 times increase of iron bioavailability, and a strong negative correlation between them was also reported in Experiment 3 Inheritance Study. However, in the bean and rice studies, phytate concentration was not correlated with iron bioavailability (Glahn et al., 2002b; Welch et al., 2000). Meanwhile, a soy-protein study indicated a meaningful increase of iron bioavailability can only occur with a 90 % reduction of phytate concentration (Hurrell et al., 1992).

The reasons for the different conclusions might be due to difference of the varieties utilized and the PA:Fe molar ratio in different varieties or species. In the paper of Aluru et al. (2011) and in this research, the varieties were selected mainly for their differences in phytate concentration, while in the bean and rice studies selected varieties contained multiple differing traits such as polyphenols level rather than just phytate level (Glahn et al., 2002b; Welch et al., 2000). Thus, the multiple trait differences among varieties enhanced the variance of iron bioavailability and resulted in a blurring of the relationship between phytate concentration and iron bioavailability. According to a laboratory study with a simple food matrix, when PA:Fe is higher than 10:1, phytate concentration had a very limited influence on iron bioavailability (Glahn et al., 2002a). In the rice study, the varieties contained PA:Fe in a range of 13:1 to 80:1 (Glahn et al., 2002b), that were in excess of the range where iron bioavailability can be influenced by phytate concentration.

In Experiment 2 Seed Coat Study, the difference of iron bioavailability between low (1-2347-144) and normal (CDC Bronco and CDC Meadow) phytate varieties was smaller than what was presented in the Experiment1 Phytate Study. Besides experimental error of *in vitro* digestion/Caco-2 cell culture bioassay, the result might also be caused by the environmental effect and it could be explained by the PA:Fe molar ratio. Experiment 1 and 2 used the same seed sources of 1-2347-144, CDC Bronco and CDC Meadow from 2009 and

2010 Rosthern. Experiment 1 already showed that seeds from 2009 and 2010 Rosthern presented much smaller difference between low phytate line (1-2347-144) and normal phytate varieties (CDC Bronco and especially CDC Meadow), meanwhile, their estimated PA:Fe were all bigger than 12:1 with which phytate concentration might have limited effect on iron bioavailability. Thus, when breeding the higher-iron-bioavailability trait as a benefit from the low phytate technique, it might be necessary to have an assessment of PA:Fe molar ratio to estimate if there is available space for phytate concentration to affect iron bioavailability.

In Experiment 2 Seed Coat Study, polyphenols in pigmented seed coats also had a substantial influence on iron bioavailability. A similar effect of pigmented seed coats on iron bioavailability had been demonstrated in common bean, lentil, pea and other foods (DellaValle et al., 2013; Glahn et al., 2002b; Jin et al., 2009; Tako and Glahn, 2011; Troszyńska and Ciska, 2002). Meanwhile, dehulling efficiently increased iron bioavailability in the varieties with pigmented seed coat resulting in two times higher iron bioavailability in selected lentil varieties (DellaValle et al., 2013) and five to six times higher iron bioavailability in selected pea varieties in this research, respectively.

Raising the total iron concentration can be another approach to increase iron bioavailability; however, to many studies, total iron concentration and iron bioavailability did not showed a significant correlation. In this research, variety 40-10 contained greater total iron concentration, but had the lowest iron bioavailability due to its pigmented seed coat; the low phytate lines had similar total iron concentration to the normal phytate varieties, but had significantly greater iron bioavailability due to their phytate level. In the lentil study, iron concentration was not correlated with iron bioavailability in both situations of dehulled and whole seeds (DellaValle et al., 2013). Additionally, no correlation was found between iron concentration and iron bioavailability in bean (Welch et al., 2000), rice (Glahn et al., 2002b), maize (Pixley et al., 2011), and peas (Inheritance Study of this thesis).

Thus, instead of only increasing iron concentration, more studies now focus on

increasing the accumulation of high-bioavailable iron. Ferritin has a relatively higher iron bioavailability due to its different absorption mechanism from non-heme-non-ferritin iron (such as ferric citrate and ferrous sulfate), although it is considered as non-heme iron in both animals and plants (Theil and Briat, 2004). Aluru et al. (2011) and Drakakai et al. (2005) reported that the transgenic maize lines with the soybean ferritin gene could also increase the iron bioavailability by 2 times. The same soybean ferritin gene was also introduced into rice and resulted in a 2 times higher iron concentration, but iron bioavailability was not directly tested (Lucca et al., 2001). Bodnar et al. (2013) tried to increase iron bioavailability by increasing heme iron level in maize endosperm. They overexpressed a maize globin gene to increase the hemoglobin level, however, they determined that iron bioavailability did not differ between the transformed and untransformed seeds.

Currently, many studies are focused on increasing the promoters of nutrients or decreasing the anti-nutritional factors. Lucca et al. (2001) created rice lines with an overexpressed cysteine gene (rgMT-gene), resulting in a seven-time increase of cysteine, a promoter of iron bioavailability. However, to the most prevalent promoter ascorbic acid, limited success has been found in increasing iron bioavailability by increasing ascorbic acid level in crops.

As a quantitative trait, the inheritance of iron bioavailability is complex (Anderson et al., 2012; Bañuelos et al., 2008; Kalgaonkar et al., 2008). It would be valuable to test the inheritance of bioavailability of nutrients in biofortified crops. The heritability of maize iron bioavailability was estimated between 0.55 and 0.65 by testing hybrids grown in two or three locations (Pixley et al., 2011). Another iron bioavailability study found ten QTLs in the B73 × Mo17 recombinant inbred maize population (Lung'aho et al., 2011). In the Inheritance Study, the iron bioavailability was highly correlated with phytate-phosphorus, and followed a bimodal pattern, which might show similar QTL as phytate-phosphorus in PR-15 RILs. Identification of QTLs of iron bioavailability will aid in relieving iron deficiency and anemia worldwide.

In this project, phytate and polyphenols had significant effects on iron bioavailability in pea seeds; however, CDC Meadow contained some unknown factors that contributed to relatively higher iron bioavailability. In the Phytate Study, at three out of four environments CDC Meadow contained higher iron bioavailability than CDC Bronco and CDC Golden. In Chicken Study 3, the CDC Meadow diet resulted in the lowest body weight of chicken. Phytate is packed in protein bodies which vary in their density of packing (Lott et al. 1995). Differences in packing of phytate might be one of the reasons that CDC Meadow had similar phytate level, but higher iron bioavailability compared to the other normal phytate varieties. Also, CDC Meadow might contain some unknown nutritional components that differ from the other normal phytate varieties.

In the Phytate Study, the differences in iron bioavailability between the low and normal phytate varieties in 2009 and 2010 at Rosthern were much smaller than that at the other two locations. In the Seed Coat Study, the pea samples were from Rosthern 2009 and 2010, and CDC Meadow was also one of the varieties; this might explain why there were no significant differences in iron bioavailability between the low and normal phytate varieties.

PR-15, derived from 1-2347-144 × CDC Meadow, was designed primarily for the study of phytate-phosphorus, not for the iron bioavailability trait. Meanwhile, the PR-15 lines grown at Rosthern in 2012 were chosen for the Inheritance Study because of greater seed availability compared to the Sutherland 2012 location. These coincidences made Rosthern the location that was mainly studied in this project, which seemed to have some unknown factors that minimized the difference of iron bioavailability between low and normal phytate pea varieties in the Phytate Study and the Seed Coat Study, and resulted in the imperfect bimodal distribution and correlation between phytate-phosphorus and iron bioavailability in the Inheritance Study.

7.2 Conclusion and future study

In this study, the low phytate pea lines (1-150-81 and 1-2347-144) contained significantly

higher iron bioavailability and inorganic phosphorus, lower phytate-phosphorus and a similar amount of total iron concentration compared to their progenitor CDC Bronco and other normal phytate pea varieties. Growing conditions (environment) also significantly affected iron bioavailability. The *in vivo* chicken study did not show similar result as the *in vitro* study, in that the low phytate diets had no significant effect on bird body weight and hemoglobin concentration, due to the unexpected iron-enriched ingredients, particularly the limestone and dicalcium-phosphate. Separate from the influence of phytate, seeds with pigmented seed coat also displayed reduced iron bioavailability compared to seeds with non-pigmented seed coats. Iron bioavailability was highly correlated with phytate concentration in PR-15, showing a bimodal distribution, suggesting a pleiotropic effect of the single recessive gene controlling phytate-phosphorus concentration.

Improving nutritional value of crops is of increasing importance, especially nutritional bioavailability rather than just concentration in the food. Knowledge of the inheritance of bioavailable nutrients will facilitate breeding. Increasing iron bioavailability has been successfully achieved by decreasing phytate and polyphenol concentration in maize, lentil and pea, and by introducing the heat-tolerant phytase gene or ferritin gene in rice and maize.

Potential future researches related to this study include the following.

1) Further study of the inheritance of iron bioavailability, more field trials in different locations (such as SPG or Outlook) and years can be conducted to make it more accurate, QTL analysis can conducted for the iron bioavailability trait in PR-15. Other recombinant inbred populations which are not only associated with the phytate trait can be evaluated to generate a broader understanding of iron bioavailability in field pea.

2) A chicken study can be conducted with a re-designed diet prepared to exclude the interference of ingredients and environments with extra iron.

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9.0 Appendices

Table 9.1. IN-P (inorganic phosphorus, ppm) of two low phytate lines (1-150-81, 1-2347-144) and three normal phytate varieties (CDC Bronco, CDC Golden and CDC Meadow) at 2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern.

Environment / Name	2009 SPG	2009 Outlook	2009 Rosthern	2010 Rosthern	Variety Mean	SEM
1-150-81	2083 a	2764 a	2081 a	2749 a	2420 a	195
1-2347-144	1984 a	2527 b	1897 a	2579 a	2247 b	178
CDC Bronco	584 b	616 c	591 b	600 b	598 c	7
CDC Golden	694 b	692 c	649 b	630 b	666 c	16
CDC Meadow	769 b	684 c	747 b	585 b	696 c	41
Location Mean	1223 b	1457 a	1193 b	1428 a		
	p<.0001	p<.0001	p<.0001	p<.0001		

Note: Means for variety, year and their interaction were separated by Tukey's Mean Comparison with a significance level at P≤0.05. SEM, standard error of the mean.

Table 9.2. PA-P (phytate-phosphorus, ppm) of two low phytate lines (1-150-81, 1-2347-144) and three normal phytate varieties (CDC Bronco, CDC Golden and CDC Meadow) at 2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern.

Name \ Environment	2009 SPG	2009 Outlook	2009 Rosthern	2010 Rosthern	Variety Mean	SEM
1-150-81	1432 b	1468 c	1603 b	1832 c	1584 c	91
1-2347-144	1285 b	1339 c	1571 b	1719 c	1478 d	101
CDC Bronco	2574 a	2918 a	2624 a	2804 a	2730 b	80
CDC Golden	2849 a	2905 a	2694 a	2963 ab	2853 a	58
CDC Meadow	2667 a	2743 b	2768 a	2570 b	2687 b	44
Location Mean	2161 c	2275 b	2252 bc	2378 a		
	p<.0001	p<.0001	p<.0001	p<.0001		

Note: Means for variety, year and their interaction were separated by Tukey's Mean Comparison with a significance level at $P \leq 0.05$. SEM, standard error of the mean.

Table 9.3. FECON (total iron concentration, ppm) of two low phytate lines (1-150-81, 1-2347-144) and three normal phytate varieties (CDC Bronco, CDC Golden and CDC Meadow) at 2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern.

Environment / Name	2009 SPG	2009 Outlook	2009 Rosthern	2010 Rosthern	Variety Mean	SEM
1-150-81	42.7 a	39.3 a	40.9 bc	38.1 b	40.3 a	1.0
1-2347-144	40.6 a	37.4 ab	40.9 bc	40.4 ab	39.8 a	0.8
CDC Bronco	43.1 a	34.9 b	40.0 c	39.4 ab	39.3 a	1.7
CDC Golden	44.7 a	35.2 b	44.2 a	42.1 a	41.6 a	2.2
CDC Meadow	44.2 a	38.4 ab	43.7 ab	40.5 ab	41.7 a	1.4
Location Mean	43.1 a	37.0 b	41.9 a	40.1 ab		
	p=0.3406	p=0.0995	p=0.0523	p=0.2259		

Note: Means for variety, year and their interaction were separated by Tukey's Mean Comparison with a significance level at $P \leq 0.05$. SEM, standard error of the mean.

Table 9.4. FEBIO (iron bioavailability, ng ferritin/mg protein) of two low phytate lines (1-150-81, 1-2347-144) and three normal phytate varieties (CDC Bronco, CDC Golden and CDC Meadow) at 2009 SPG, 2009 Outlook, 2009 Rosthern and 2010 Rosthern.

Environment / Name	2009 SPG	2009 Outlook	2009 Rosthern	2010 Rosthern	Variety Mean	SEM
1-150-81	30.7 a	17.9 a	21.9 a	25.2 a	23.9 a	2.7
1-2347-144	32.7 a	19.5 a	19.7 ab	26.8 a	24.7 a	3.2
CDC Bronco	16.4 bc	9.5 c	14.0 c	19.7 b	14.9 c	2.1
CDC Golden	13.2 c	10.2 c	15.4 c	18.1 b	14.3 c	1.7
CDC Meadow	19.8 b	14.3 b	18.2 b	18.8 b	17.8 b	1.2
Location Mean	22.6 a	14.3 c	17.8 b	21.7 a		
	p<.0001	p<.0001	p=0.0004	p<.0001		

Note: Means for variety, year and their interaction were separated by Tukey's Mean Comparison with a significance level at $P \leq 0.05$. SEM, standard error of the mean.