INCREASING PHOSPHORUS EFFICIENCY: AN INVESTIGATION OF PHOSPHORUS UPTAKE MECHANISMS IN THE RHIZOSPHERE OF WHEAT

By

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CHAPTER I

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INTRODCUTION

By 2030, the global demand for cereal crops is predicted to rise by 31% to 2.83 billion metric tons (Bruinsma, 2003; WOAB, 2011). This demand will largely come from areas of the world with poor growing environments and infertile soils. In some of these areas, farmers have limited resources to add fertilizer phosphorus (P) to increase their crop yields (Runge-Metzger, 1995). Thus, in order to meet the food demand, more land will need to be put under production. This subjects highly erodible land and fragile ecosystems to exploitation that begins a spiral of environmental degradation (Young, 2005).

In developed countries, addition of fertilizer (in the form of chemical fertilizer or manure) accounts for 30 to 50% of crop yield (Stewart et al., 2005). Only 10-20% of the fertilizer being applied is recovered during the growing season (Mclaughlin et al., 1988; Sharpley, 1986). Unavailability of these nutrients decreases efficiency and leads to both economic losses and environmental deterioration.

The phosphate rock reserves that agriculture relies on are depleting and estimated to reach near exhaustion in the next 40-60 years. With this depletion, P prices are expected to rise even higher (Figure 1.1) and will affect the cost of producing food (Steen, 1998).

Additionally, overuse of P fertilizer has led to an increase in potential for losses to surface waters (Sharpley et al., 1994). This leads to eutrophication in surface waters and degrades the quality of water bodies (Cassman, 1999).

Even though many soils have enough P to supply plant needs for many years (Goldstein et al., 1993), P is limiting on 30% of arable crop land (Korkmaz et al., 2009; Vance et al., 2003). This P limitation is due to the P being present in typically non-labile forms. In addition, although total soil P levels vary from 100 to 1500 mg kg^{-1}, only a portion of this is typically plant available as indicated by agronomic soil tests such as Mehlich 3 (M3), in which "optimum" P level is 32 mg kg^{-1}. In other words, there is a very large pool of soil P that is mostly "untapped" by agronomic crops.

For forest plants, however, this P pool that is typically non-labile for agronomic crops is more labile. Consider that although P solubility is very low in native forest and grassland soils, such native plants are still able to produce a large amount of biomass (Chen et al., 2003). In other words, these plants have the "machinery" to access or dissolve soil P pools that would be non-labile to agronomic crops such as wheat.

While P use efficiency has been examined in wheat, the studies have focused on variation in P efficiency between plants (Bhadoria et al., 2002; Föhse et al., 1991;

Gardiner & Christensen, 1990; Manske et al., 2002) rather than root-rhizosphere soil interactions that contribute to the recognized efficiency.

The purpose of this project is to evaluate the phosphorus (P) use efficiency of various wheat cultivars and to determine the mechanisms that plants use to obtain tightly-bound P in the soil rhizosphere. Wheat rhizosphere soil will be analyzed for known plant P-uptake mechanisms such as organic acids and phosphatases. The rhizosphere soil will also by analyzed for changes in soil pH and soil P fractions.

Potential Impact

The immediate impact from this project will be reductions in P fertilizer rates to wheat for producers in the Southern Great Plains who choose the more P efficient cultivars. This will be achieved through an assessment of P use efficiency among wheat cultivars, the results of which will be directly disseminated to producers and breeders. This will be especially important to producers with low agronomic soil P levels or those who want to apply less (or zero) P to soils. The second, longer-term impact will be from determining P uptake mechanisms among P efficient cultivars. That information will be used by plant geneticists in identifying genes that control such mechanisms, which will then be used by wheat breeders for incorporation of high P use efficiency traits into new varieties.

LITERATURE REVIEW

Plant P Use

Phosphorus is an essential nutrient in many biological processes. It has been called “the key to life” because it is present in every living cell as a component of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the cell nucleus (Troeh and Thompson, 1993a). Phosphorus is also essential in plant respiration. Hydrolysis of the terminal phosphate group in adenosine triphosphate (ATP) during its conversion to adenosine diphosphate (ADP) provides the energy needed for many plant processes (Noggle and Fritz, 1983).

Phosphorus is mobile in plants. It can move from older parts of the plant to younger parts in order to meet the nutrient requirements for new growth. However, if the soil is low in available P, the plant will not absorb enough P to increase vegetative growth (Gardner et al., 1985).

Soil P

Of the total P found in soil, less than 0.01% is available for plant uptake. Phosphorus in soils can be separated into three pools; solution P, active P, and fixed P. The solution P pool is usually in the orthophosphate form (HPO_4^{2-} and $H_2PO_4^-$), making it readily available for plant uptake. However, this pool is very small compared to the total P in the soil. The active P pool is composed of readily soluble inorganic P and easily mineralized organic P. The active P pool releases P into solution as the solution P pool dissipates. The fixed P pool consists of relatively insoluble inorganic P that releases P into the active pool, although not in a large quantity (Busman et al., 2002).

Phosphorus is found in three general forms in the soil; organic P, calcium (Ca)-bound inorganic P, and iron (Fe)- or aluminum (Al)-bound inorganic P (Brady and Weil, 2008). Organic P forms include phospholipids, nucleic acids, and inositol phosphates (Tisdale and Nelson, 1975). The fraction of soil organic P is small compared to inorganic P.

Soil pH plays a major role in the form of inorganic P found in soils. In high pH soils, Ca is very abundant and P readily reacts with it to form relatively insoluble calcium phosphate compounds. However, if the pH decreases, these calcium phosphate compounds become increasingly soluble. Iron- or aluminum-bound P is found predominately in acid soils. The insoluble Al or Fe-phosphate compound then precipitates or adsorbs to soil particles, rendering it even less available to plants (Bohn et al., 2001).

Phosphorus can also be fixed to soil particles through interaction with valence unsatisfied edges on soil minerals. In this reaction, phosphate ions either replace a hydroxyl group from an aluminol or form a clay-Ca-phosphate linkage (Tisdale and Nelson, 1975). The ability of a soil to fix P changes with varying amounts and types of clay minerals, organic matter, and variation in soil pH(Brady and Weil, 2008).

P Fertilizer

Because of its importance in maintaining an economically viable crop harvest, P fertilizer is used in many farming systems around the world. Phosphorus is applied as both inorganic chemical fertilizer and/or animal manure.

The three most common types of P chemical fertilizers are triple super phosphate, mono-ammonium phosphate (MAP), and di-ammonium phosphate (DAP) (Beegle,). Inorganic P fertilizers are formed by extracting phosphate rock from the earth and reacting it with sulfuric acid, forming phosphoric acid and calcium sulfate. The mixture is then further treated by filtration and washing which separates the calcium sulfate from the phosphoric acid. The phosphoric acid is then concentrated using a vacuum evaporator. This concentrated P source is then processed to the desired particle size. In the case of ammonium phosphates, phosphoric acid is mixed with ammonium sulfate or ammonium nitrate in order to create a fertilizer that supplies both P and N (Phillips and Boylan, 1963).

Organic P fertilizer in the form of animal waste includes manure slurries, litter, effluent, and compost. Although nutrient concentrations vary among manure sources, 90% of P found is typically available the first year it is applied (Zhang,). This is due to P being mainly in the inorganic form when found in animal waste.

In general, when a P fertilizer is added to the soil, water enters the granule and begins to dissolve it. This solution then begins to move out into the soil away from the granule. When in the soil solution, the P can be adsorbed to soil particles, reacted with Al, Fe, and Ca depending on pH, immobilized by soil microorganism, or remain in the soil solution (Sylvia et al., 2005; Tisdale & Nelson, 1975).

Phosphorus Use Efficiency

Phosphorus efficiency of various plant species has become a widely studied subject in recent years. Hammon et al. (2004) describes plant P use efficiency as being

composed of four areas; early signaling events, morphological, metabolic, and physiological responses. Early signaling events include ribo-regulators which regulate gene expression under various conditions. Morphological responses include increasing root-shoot ratio or increasing growth of lateral roots. Plants can alter metabolism by alternative photosynthetic and respiratory pathways. Physiological responses to P stress focus on modifying rhizosphere conditions in order to increase P uptake; this includes the exudation of organic acids and phosphatase enzymes. The proposed study will focus on physiological responses due to the fact that even if a plant is efficient at utilizing P, in low P soil it still must have the ability to uptake P in order to survive.

Determination of plant P efficiency is achieved by quantifying the plant tissue P concentration per mass of plant yield (Batten, 1992). A phosphorus efficiency index can also be determined using this method by taking the biomass production per unit of P concentration within the plant. Plant yield can be either grain yield or biomass yield. Plants that have high biomass yields with low P concentrations in low P soils are considered P-use efficient because of their low internal P requirement. Plants with high biomass and high P concentrations in low P soils are considered P-uptake efficient, utilizing various strategies in order to increase uptake in low P environments (Föhse et al., 1991).

Korkmaz et al. (2009) found considerable difference between ten wheat genotypes growing in a calcareous soil, with the efficiency index ranging between 1.5- to 6.9 g^2 (total P content)$^{-1}$. In a study comparing the efficiency of various durum and bread wheats, Ozturk et al. (2005) found that shoot dry weight varied significantly between wheat genotypes at low $NaHCO_3$-extractable P levels (20 mg kg^{-1}). Bread wheat shoot

dry weight was between 345 mg to 629 mg while durum wheat varied between 442 mg and 688 mg. Similar differences were seen by Osborne and Rengle (2002) in their study of Australian wheat cultivars with shoot dry weight yields ranging from 108 mg to 278 mg in P deficient soils.

Root Exudation

Plants uptake most mineral nutrients from the rhizosphere; i.e. the thin layer (0-2mm) of soil surrounding plant roots. In response to abiotic and biotic stresses, including nutrient deficiency, plants exude many different compounds into the rhizosphere. Root mechanisms of exudation can take place via three different processes; diffusion, ion channel, and vesicle transport. Diffusion is passive and occurs when there is a lower concentration of the substance in the rhizosphere compared to inside the root cells. Ion channels actively control the release of specific chemicals such as low-weight organic acids, playing the role of "gate keeper" for particular substances. Ion channels open and close in response to external signals. Once an ion channel is open, only specific substances can pass through the channel leaving the root. The channel pore size and inner charge determine its specificity (Taiz and Zeiger, 2010). Vesicle transport is considered an active mechanism for exudation in that it actively transports substances out of the cell. Transport vesicles function by surrounding a substance and moving it to a new location. The vesicle then fuses with the plasmas membrane at the new location and releases the substances out of the cell. Vesicle transport is used in exudation of high-molecular weight substances such as phosphatase enzymes (Bertin et al., 2003).

Organic Acids

Plants are able to access recalcitrant soil P by a variety of mechanisms, depending on soil chemical properties. Plant roots can excrete low molecular weight organic acids (LMWOA) that can chelate metals that are bound with P. Low molecular weight organic acid's also can compete with phosphate ions for adsorption sites on soil particles. Both of these methods release P into solution which makes it available for plant uptake (Hocking, 2001).

Plant exudation of organic acids when grown in P deficient soils has been reported for many different species including white lupin (*Lupinus albus L.),* pigeon pea (*Cajanus cajan (L.) Millsp),* rice (*Oryza satuva L.),* and chickpea (*Cicer arietinum L.)* (Dinkelaker et al., 1989; Hocking, 2001; Ishikawa et al., 2002). However, most of the studies have focused on white lupin, with very little being reported about wheat. Varying amounts of LMWOA have been found in plant rhizosphere; citric, malic, malonic, and oxalic are usually the most abundant.

In a study to determine organic acid synthesis and exudation in white lupin roots, Neumann et al. (1999) found the root tissue concentration and root exudation of citric and malic acids increased under P deficiency. Root tissue concentration of citric acid increased from 2.53 µmol (g fresh weight (FW))$^{-1}$ to 8.75 µmol (g FW)$^{-1}$ with root exudation increasing from .02 µmol h^{-1} (g FW)$^{-1}$ to .09 µmol h^{-1} (g FW)$^{-1}$. Malic acid tissue concentration increased from 6.13 µmol (g FW)$^{-1}$ to 7.17 µmol (g FW)$^{-1}$ with root exudation increasing from .08 µmol h^{-1} (g FW)$^{-1}$ to .61 µmol h^{-1} (g FW)$^{-1}$**.**

Keerthisingh et al. (1998) reported that citric acid levels excreted from white lupin without phosphate supply was 4.59 mmol $h^{-1}cm^{-1}$root while plants that had some phosphate supply exuded 1.48 mmol $h^{-1}cm^{-1}$root, a threefold increase when grown without P. These significant responses indicate that organic acid exudation is indeed a plant mechanism for coping with P stress.

pH Change

Another major mechanism of P uptake in low P soils is soil pH modification by plant roots. Plants modify the rhizosphere pH by net excretion of H^+ or HCO_3^- which is brought about when cation/anion uptake is not in balance (Grinsted et al., 2011). This decrease in rhizosphere pH causes acid-soluble forms of inorganic P to become available through dissolution. Hedley et al. (1982) found in a study on rape (*Brassica Napus Emerald*), that as the rhizosphere soil pH decreased from 6.4 to 4.5, P desorbing into solution increased from 0.14 µmol g^{-1} soil to 1.5 µmol g^{-1} soil. This significantly increased the amount of P available for plant uptake. Gahoonia and Nielsen (1992) observed an overall 20% depletion from all fractions of soil P when the pH decreased from 6.7 to 5.5.

Organic acids also have an effect on the pH of plant rhizosphere soil. However, their effect on pH is small compared to H^+ and HCO_3^- excretion unless the soil exhibits P deficiency (Gahoonia and Nielsen, 1992).

Phosphatase Enzymes

In regards to soil organic P, some plant roots are able to produce phosphatase enzymes. These enzymes remove phosphate groups from phytic acid (the most common

form of soil organic P), rendering it plant available as phosphate anions. This is especially important considering that a large amount of inorganic P fertilizer applied is immobilized by microorganisms into organic P (Richardson et al., 1994).

It has been shown that plants produce more phosphatase enzymes when under P deficiency than when P is sufficient (Dinkelaker and Marschner, 1992). In a study comparing extracellular acid phosphatase activity between two wheat cultivars grown in a nutrient solution, Ciereszko et al. (2011) found that after three weeks of growth, acid phosphatase activity increased from around 1 µmol paranitrophenol (*p*NP) g^{-1} FW min^{-1} to about 5 µmol *p*NP g^{-1} FW min^{-1}. There was also an increase in intracellular acid phosphatase activity for both wheat cultivars when under P stress.

Tarafdar (1987) found that there was a decrease in organic P in the rhizosphere soil of clover and wheat compared to the bulk soil, with organic P levels dropping from around 35 mg 100 g^{-1} soil to around 10 mg 100 g^{-1} soil. This was in conjunction with increasing phosphatase levels, expressed in enzyme units (E.U.)- the amount of enzyme that hydrolyses 1.0 µmol *p*-nitrophenyl phosphate/min, from 0.5 E.U. x 10^{-3} to 3.0 E.U. x 10^{-3} for wheat and 0.5 E.U. x 10^{-3} to 1.0 E.U. x 10^{-3} for clover. There is a strong correlation between this increase in phosphatase activity and the depletion of organic P in the rhizosphere soil.

While much is still to be studied in plant-rhizosphere interactions, it is apparent that much can be gained by evaluating agronomic crops for nutrient efficiency. Applying the aforementioned techniques and information to study the rhizosphere will

give us an idea of how much these physiological adaptations contribute to plant P-efficiency.

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TABLES AND FIGURES

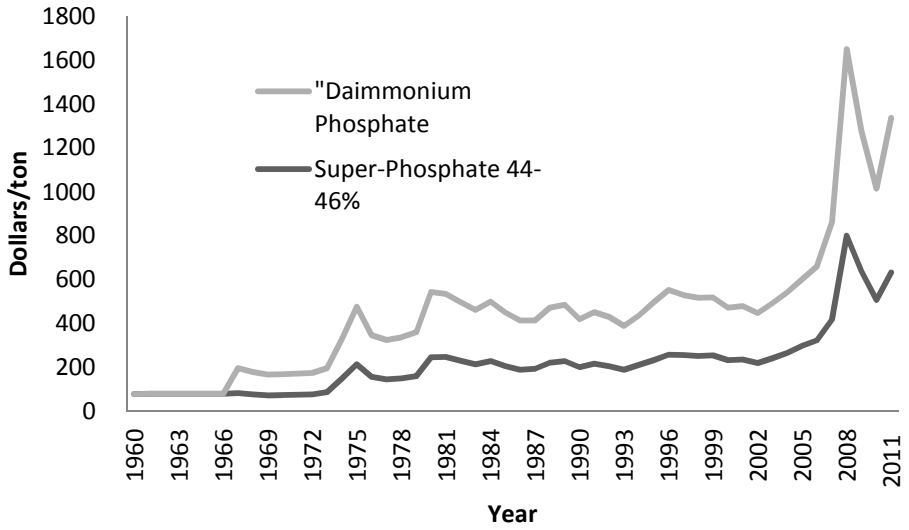

Figure 1.1 Price of selected phosphate fertilizers from 1960-2011 (USDA Cost, 2011)

CHAPTER II

COMPARISON OF PHOSPHORUS EFFICIENCY AMONG VARIOUS WINTER WHEAT ASSESSIONS GROWN IN ACID AND CALCAREOUS SOILS

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ABSTRACT

The phosphate reserves society relies on are decreasing in both quantity and quality. It is therefore very important to increase the efficiency with which our agricultural systems cycle P. The objective of this study was to evaluate twenty-two winter wheat accessions for phosphorus (P) efficiency and subsequently characterize them as P-uptake and P-utilization efficient when growing in both acid and calcareous soils. The wheat accessions were grown in low, medium, and high P acid and calcareous soils for 28 days in a growth chamber in two different sized pots. At the end of the growing period, plants were harvested to measure biomass and P-uptake for each accession. Accessions varied in the ability to efficiently utilize and uptake P. For the acid soil, six accessions were characterized as predominantly P-uptake efficient and 3 accessions as P-utilization efficient. In the calcareous soil, six accessions were characterized as primarily P-uptake efficient and four as P-utilization efficient.

INTRODUCTION

Demand for phosphorus (P) is steadily rising and the P reserves we rely on are decreasing in both quantity and quality (Steen, 1998). These reserves are predicted to reach near exhaustion within the next 46-60 years (Stewart et al., 2005). In addition, global demand for cereal crops is estimated to increase 31% by 2030 (Bruinsma, 2003; WOAB, 2011). To meet this demand it is imperative that resource efficiency, especially P efficiency, of all major crops be increased. Failing to do so could lead to the exploitation of marginal land and the resulting negative environmental and social consequences.

Of the 17.8 Mt P_2O_5 applied as fertilizer worldwide, wheat utilizes 16.4%; second in consumption only to fruits and vegetables combined (Heffer, 2009). Improved P efficiency for wheat accessions not only leads to better conservation of this natural resource, but also aids in protecting the environment from the eutrophication of surface waters. If producers can apply less P and still achieve the same yields, there will be less chance of P runoff into surface waters and the subsequent water quality implications.

Improving P efficiency of wheat can be done either by increasing P-utilization efficiency or P-uptake efficiency (Batten, 1992; Osborne and Rengel, 2002). Phosphorus-utilization efficiency is distinguished by a plant's ability to achieve high biomass with low amounts of P in plant tissue. In order to achieve P-use efficiency, plants respond with various metabolic, morphological and physiological responses such as using alternative metabolic pathways and increased root-shoot ratio (Hammond et al., 2004). On the other hand, P-uptake efficiency involves the ability to achieve high yields in low available P environments by extracting typically non-labile phosphorus (Föhse et al., 1991). Plants achieve this by exuding substances

such as organic acids and phosphatase enzymes or by adjusting rhizosphere soil pH (Dinkelaker et al., 1989; Hedley et al., 1982; Hocking, 2001; Tarafdar, 1987). These strategies desorb and solubilize P, which makes it more available for plant uptake.

Wheat P efficiency studies have focused primarily on identifying differences in yield between various accessions with little attention paid to distinguishing between P-uptake and P-use efficiency. The objective of this study was to identify and differentiate P-uptake versus P-use efficiency in winter wheat germplasm. Successful attainment of this objective would allow one to identify phenotypes with elevated P-uptake efficiency for further studies on P-uptake mechanisms by P-uptake efficient plants. The ultimate goal would be to introgress genes for high P-uptake efficiency into new accessions.

METHODS

Wheat Accessions

Because P efficiency can be mechanistically linked (e.g., organic acid exudation) to aluminum tolerance in acidic soil, we constructed a panel of 22 winter wheat accessions with a wide range of expected responses to aluminum toxicity and acidic soil. Eight accessions, numbered 1 to 8 in this study, were chosen for their putative field tolerance in naturally acidic field sites near Enid, OK (conditions described previously by Edwards et al., 2012) in the absence of a resistance allele at the Al-activated malate transporter (ALMT1) locus on chromosome 4DL (Bai, 2011, personal communication).

Accessions 9 to 13 represented two pairs of lines near-isogenic (97% genetic similarity within pairs) for a gene inherited from ‘Atlas 66’ that confers Al-inducible malate exudation from root tips but not constitutive release of phosphate (Carver et. al, 1993; Tang et. al, 2002).

OK91G103 and OK91G107 are Al-tolerant and Al-sensitive near-isolines of the cultivar ‘Chisholm’, respectively, whereas OK91G105 and OK91G108 are Al-tolerant and Al-sensitive near-isolines of the cultivar ‘Century’.

Accessions 13 to 22 were chosen to represent contemporary hard red winter (HRW) germplasm, with or without putative acid-soil tolerance, but primarily featuring parent-offspring relationships that proportionately diminish genetic differences. Accession 14, ‘Iba’, is an acid-soil susceptible offspring of the tolerant accession 13, Duster, which also contains an Al-resistant marker allele of *ALMT1* (Edwards et al., 2012). Likewise, accession 16, ‘Ruby Lee’, is a susceptible offspring of the acid-soil tolerant accession 15, ‘Endurance’, which contains an Al-resistant marker allele of *ALMT1* (Carver et al., 2006). In addition to the acid-soil susceptible hallmark HRW wheat cultivar ‘Karl 92’ (accession 17), accession 18 (‘Jagger’, Sears et al., 1997) is a parent of accession 19 (‘Fuller’), and a grandparent of accession 20 (‘Garrison’). Jagger (+*ALMT1*) and Garrison (-*ALMT1*) are highly tolerant of acidic soils. Lastly, accessions 21 and 22 are experimental lines featuring low-pH tolerance in the absence of a functional allele of *ALMT1*.

Considering the entire panel, accessions 10, 12, 14, 16, 17, and 19 (Chisholm-S, Century-S, Iba, Ruby Lee, and Fuller) are considered intolerant of acidic soil under field conditions. Of the remaining (tolerant) accessions, accessions 9, 11, 13, 15, and 18 (Chisholm-T, Century-T, Duster, Endurance, and Jagger) are supported by experimental evidence or molecular marker data to exhibit Al-induced release of organic acids.

Soil Analyses

Two soils were used during this experiment; an acid and a calcareous soil. Before use, soils were dried at 65° C, sieved to 2 mm, and analyzed for pH, NO_3-N, K, SO_4-S, Ca, Mg, Fe, Zn, B, Cu, Total P, and extractable P. Soil pH was measured with a combination electrode in a 1:1 soil to water suspension (Thomas, 1996). Nitrate nitrogen (NO_3-N) was analyzed with 2.5 g of soil and 10 mL of 1.0 M KCl. This mixture was shaken for 15 minutes and the extract was analyzed for NO_3-N using a Lachat Quickchem 8000 automated flow-injection analyzer (Mulvaney, 1996). The SO_4-S was measured by extracting 10 g of soil with 25 mL of 0.008 *M* calcium phosphate, shaking for 30 minutes subsequently analyzing with inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Spectro Ciros, Mahwah, NJ) (Schulte and Eik, 1988). Iron, Zn, B, and Cu were extracted by taking a 10 g soil sample and shaking with 20 mL of DTPA-Sorbitol (0.005 *M* DTPA, 0.1 *M* triethanolamine (TEA), 0.01 *M* $CaCl_2$, and sorbital) for 2 hours. The extract was then analyzed by ICP-AES (Lindsay and Norvell, 1978). Agronomic K, Ca, Mg, and P were measured by extracting a 2.0g soil sample with 20 mL of Mehlich-3 (M3) solution (0.015 *M* NH_4F, 0.2 *M* CH_3COOH, 0.25 *M* NH_4NO_3, 0.013 *M* HNO_3, 0.001 *M* EDTA) and shaking for 5 minutes (Mehlich, 1984).The resulting solution was filtered through a #2 Whatman filter paper and then analyzed by ICP-AES. For the calcareous soil, agronomic P was extracted by using 2.0 g of soil and 40 mL of 0.5 *M* $NaHCO_3$, shaking for 30 minutes, and then filtration through a #2 Whatman filter paper. The resulting solution was then analyzed on an ICP-AES (Olsen et al., 1954).Total P for each soil was measured by performing an acid digestion on 0.5 g of soil according to the EPA 3050 method; solutions were then analyzed by ICP-AES (EPA, 1986). Soil texture was determined by the hydrometer method (Gee and Baude, 1986). To

measure exchangeable aluminum, 5g of soil was shaken with 20 mL of 1.0 *M* KCl for thirty minutes, filtered, and then analyzed in ICP-AES (Page et. al, 1982).

Each accession was grown in the acid and calcareous soils. The calcareous soil used was a Lucien sandy loam (loamy, mixed, superactive, thermic, shallow Udic Haplustolls) from Guymon, Oklahoma; the acid soil utilized was a Richfield clay loam (fine, smectitic, mesic, Aridic Argiustolls) from Waurika, Oklahoma (Table 2.1).

Growth Conditions

Wheat accessions were grown in a growth chamber for twenty-eight days after emergence. Four seeds were planted in each pot and were thinned to one plant per pot after emergence. Daytime temperatures were 25 ^{o}C and nighttime temperatures were 20 ^{o}C. The plants received 16 hours of daylight at a light intensity of 200 µmoles m^{-2} per s. Each pot was placed in a water reservoir containing a 1400 mg L^{-1} potassium nitrate (KNO_3) fertilizer solution. This was done to allow the soil to remain near constant field capacity while ensuring that the plants would not experience nitrogen deficiency. Both soils contained adequate K and micronutrients (Table 2.1).

At the end of the growing period, shoot biomass was harvested and dried at 85 ^{o}C for 24 hours to determine plant dry weight. Due to low biomass production, replications of each accession from the low P soil were composited, ground, and digested (Jones and Case, 1990), and the resulting solution analyzed by ICP-AES to determine P concentration. To calculate mg P uptake, the measured P concentration (mg P kg $biomass^{-1}$) was multiplied by the combined biomass (kg) and then divided by the number of composited plants (four plants) (eq.1).

(1) $P\ uptake\ (mg) = \frac{Plant\ P\ concentration \times total\ plant\ biomass}{number\ of\ plants\ in\ composite}$

Each accession was replicated four times in a randomized complete block design for two experiments. The first study utilized "large" growing pots (Pot_L) with 200 g of soil and the second study had "small" pots (Pot_S) with 62 g of soil. This was done to differentiate between accessions possessing P-uptake and P- utilization efficiency due to the differing amounts of total P present as a function of volume.

Statistical Analysis

Data was analyzed using the GLM procedure of the SAS software package (SAS Institute, 2009). Contrast statements were used to analyze the differences between accession biomass using an alpha value of 0.10.

RESULTS AND DISCUSSION

Wheat biomass and p-uptake

Background soil P concentrations were considered very low for each soil (Table 2.1). For wheat, soils with M3 extractable P concentrations below 15 mg P kg $soil^{-1}$ or Olsen extractable P concentrations below 10 mg P kg $soil^{-1}$ are considered to be very low in available phosphorus (Sawyer et al., 2011). In the low P acid soils, plant biomass varied from 64 mg to 403 mg in Pot_L and from 45 mg to 153 mg in Pot_S (Table 2.2). In the low P calcareous soil, biomass varied from 203 mg to 489 mg in Pot_L and from 43 mg to 155 in Pot_S (Table 2.3). Both Pot_L and Pot_S showed significant differences between accessions grown in each respective soil at low soil P levels. In general, accessions yielded lower in the acid soil than the calcareous soil for both pots.

Accessions grown in the calcareous soil yielded on average 60% higher than those in the acid soil. This difference in yield between soils is probably due to P being found in different forms at the two soil pH levels. One accession could be exuding what is necessary to desorb and solubilize a particular P form present in one soil, while this same exudate may not be as effective in a soil with different dominant P forms (Hocking, 2001). An example could be the exudation of citric acid solubilizing calcium phosphates by decreasing the rhizosphere pH. This would not be expected to be as instrumental in an acid soil versus a calcareous soil due to the greater amount of calcium phosphate found in calcareous soils. Additionally, extractable and total P levels for the calcareous soil were slightly higher than the acid soil, possibly providing the calcareous soil a larger potential P supply. However, all accessions for each soil and experiment contained sub-critical tissue P levels at 28 days after sowing (critical P level = 0.8-0.9 % ; Reuter & J.B., 1997). Phosphorus concentrations in the plant tissue produced in the acid soil were between 0.38 and 0.85 mg P g plant^{-1} for Pot_L and were between 0.22 and 0.82 mg P g plant^{-1} for Pot_S. For the calcareous soil they were between 0.6 and 2.32 mg P kg plant^{-1} for Pot_L and 0.55 and 1.02 mg P g plant^{-1} for Pot_s.

Differences in biomass between accessions grown at the same soil P levels could be attributed to differing amounts of root hair formation (Gardiner and Christensen, 1990), various root exudation strategies (Hedley et al., 1982; Hocking, 2001), or different internal P-use efficiencies (Hammond et al., 2004). However, differences in root architecture other than root hair formation, was eliminated with the use of the relatively small pot sizes. Both Pot_S and Pot_L were filled with plant roots.

P-uptake vs. P-use efficient accessions

The large pots (Pot_L) with the acid soil contained 381 mg total P while the same sized pots of the calcareous soil contained 426 mg total P. The small pots (Pot_S) contained 118 mg total P for the acid soil and 132 mg total P for the calcareous soil. The lower mass of P in the volume of soil in Pot_S than Pot_L is critical because P is relatively immobile in low P soils since diffusion is the dominant means of movement. This is also why increased root surface area helps to increase P uptake via rooting depth or architecture (Gahoonia and Nielsen, 1997; Zhao et al., 2004). As previously mentioned, rooting depth and architecture were minimized as confounding factors within each pot type since roots filled the entire pot for both pot types. Therefore, a comparison of biomass and P uptake between the two pot sizes is ideal since the only difference between each pot size will be the volume of soil available for the roots to extract P from, and not rooting depth or architecture. Such a comparison allows one to somewhat distinguish between P-use efficiency and P-uptake efficiency. In this case, the term "accessible P" refers to the mass of P that is accessible to the roots. Since the same low P soils were used for both pot types, Pot_S possessed less accessible P than Pot_L due to a lower soil volume.

Therefore, relative to Pot_L, accessions yielding high in Pot_S were most likely to have used a P-utilization efficiency mechanism in order to achieve the highest yields with little soil P to be obtained near the root surface (due to less soil volume for the roots to exploit). This mechanism would allow the plant to produce biomass with relatively little P-uptake through the roots. Note that this does not necessarily mean that accessions that produced the highest yields in Pot_s were able to do so because of a P-utilization efficiency mechanism alone (i.e. without an efficient P uptake mechanism), but a P-utilization efficiency mechanism would be more beneficial in this scenario where there is less P accessible. The impact of a lower amount of accessible P for the two pot types is evident by the fact that biomass, plant P concentration, and P uptake were not

correlated between Pot_S and Pot_L (R^2 = 0.0003, 0.006, and 0.08 for biomass, plant P concentration, and P uptake, respectively in the acid soil; R^2 = 0.002, 0.002, and 0.0003, respectively in the calcareous soil. In other words, Pot_L and Pot_S presented different rooting and growth environments. As expected, growth in Pot_L resulted in greater biomass and P uptake compared to Pot_S (Table 2.2).

The first step in characterizing accession P-uptake efficiency is determining the relative P uptake for each accession and pot size using equation 2 below, followed by averaging relative P uptake among Pot_L and Pot_s for each accession.

$$(2)\ Relative\ P\ uptake = \frac{P\ uptake\ of\ given\ accession}{P\ uptake\ of\ accession\ with\ highest\ P\ uptake}$$

For average relative P uptake, the 75th percentile value was chosen as the threshold for identifying the most P-uptake efficient accessions; this is shown graphically in Figures 2.1 and 2.2 as the horizontal line for both acid and calcareous soils. Accessions located above the horizontal line are considered the most P-uptake efficient among the accessions tested. While this particular characterization of P-uptake efficiency is useful, it does not distinguish between accessions that are dominated by P-uptake efficiency alone from those that are both P-utilization efficient and P-uptake efficient. The ability to make this distinction requires the ability to rank accessions in P-utilization efficiency.

Pot_L displayed different trends among accessions in biomass production and P uptake than Pot_S as previously described by the lack of correlations between pot types. Due to a greater amount of soil, there was more accessible P present in Pot_L. However, a P-uptake strategy used in Pot_S would be less effective in producing higher yields unless a P-utilization efficiency mechanism is also in place. Therefore, accessions that yielded high biomass in Pot_s relative to

Pot_L and also had high relative biomass production in Pot_s, possess P-utilization efficiency. The ability of an accession to yield high biomass in Pot_s relative to Pot_L is quantified by the following:

$$(3)\ \text{Percent difference in biomass} = \left(\frac{\text{Pot}_\text{L}\ \text{biomass} - \text{Pot}_\text{S}\ \text{biomass}}{\text{Pot}_\text{s}\ \text{biomass}}\right) \times 100$$

Relative biomass for Pot_s was quantified using equation 4:

$$(4)\ \text{Relative Biomass Pot}_\text{S} = \left(\frac{\text{biomass of given accession}}{\text{biomass of accession with highest biomass}}\right) \times 100$$

The P-utilization efficient accessions were assessed using equations 3 and 4 and shown graphically in Figures 2.3 and 2.4. The vertical line in Figures 2.3 and 2.4 represents the 25th percentile value for % biomass difference (equation 3), which indicates the accessions that performed best in Pot_s relative to Pot_L are located on the left side of the line. The horizontal line represents the 75th percentile for relative biomass production in Pot_s (equation 4). Therefore, accessions located in section I (upper left) are the most P-utilization efficient.

In the acid soil, accessions 16, 6, 17, 1, 10, and 2 were the most P-uptake efficient (Figure 2.1); 20, 16, and 19 were the most P-utilization efficient (Figure 2.3). Accessions 2 and 10 were most dominated by P-uptake efficiency alone with the least interference from P-utilization efficiency (Figure 2.1). Thus, accession 16 (Ruby Lee) was among the highest in both P-uptake and P-utilization efficiency. Although accessions 1, 2, and 6 have no effective ALMT1 allele, which is typically associated with acid-soil tolerance due to root malate excretion, these cultivars have shown excellent tolerance to acid soils in field trials (Bai, 2011, personal communication). Accessions 10, 12, 14, 16, and 17 are considered susceptible to acid soils based on field trials (Carver et al., 1993; unpublished data), although 16 and 17 displayed high P-

uptake efficiency. While accession 16 is considered to be moderately susceptible to acid soils, its parent cultivar Endurance (accession15) is considered to be acid soil tolerant. Extractable Al in the acid soil was 0.3 mg kg^{-1}, which is not considered to be detrimental to many cultivars. The traditional Al tolerance mechanism is malate excretion by roots, which renders Al^{3+} non-toxic after complexation. However, these results suggest that there are other Al tolerance mechanisms that have not been characterized in these cultivars. For example, a possible scenario is that while cultivars 1, 2, 6, 10, 19, and 20 do not possess the 4DL marker related to the capacity to excrete malate for "inactivating" solution Al^{3+}, their ability to uptake or utilize P under high Al conditions would allow it to somewhat "mask" Al susceptibility. The explanation for such a potential indirect Al tolerance mechanism is that crops grown in acid soils with high levels of soil exchangeable Al suffer from reduced P uptake and root growth (Kochian, 1995; Delhaize and Ryan, 1995), which reduces the accessibility of P.

In regard to the calcareous soil, accessions 2, 4, 6, 16, 18, and 22 were the most P-uptake efficient (Figure 2.2) while 3 was the only accession showing mostly P-utilization efficiency (Figure 2.4). Among those accessions highest in P-uptake efficiency, no accession displayed a dominant P-uptake efficiency mechanism alone with little interference from P-utilization efficiency (Figure 2.2). Considering both acid and calcareous soils, accessions 2, 6, 16, and 19 were considered to be either P-uptake efficient or P-utilization efficient in both soils; among those, accession 16 (Ruby Lee) was P-uptake and P-utilization efficient in both soils.

CONCLUSSION

Accessions responded differently to low P environments. Some accessions were better able to extract P and subsequently produce high biomass, while others were able to produce high biomass under either condition where there was more or less accessible P, suggesting P-

utilization efficiency. From this, accessions were classified as either P-uptake or P-utilization efficient when grown in acid and calcareous low-P soils. In regard to acid soil conditions, several accessions that are not categorically considered to be tolerant of acid soils, displayed the highest P-uptake and P-utilization efficiency. This would suggest that aluminum tolerance was not a pre-requisite for accessions to exhibit P-efficiency in this study. Additionally, some accessions expressed high P-uptake or P-utilization efficiency and did not contain the *ALMT1* gene but showed field tolerance to acid conditions in previous experiments (Fuller (19) and Ruby Lee (16)). These accessions reportedly showed signs of acid susceptibility during emergence but eventually grew out of the condition if other environmental factors were favorable (i.e. soil moisture). The current study suggests P-efficiency mechanisms could potentially be surviving as a second line of defense for accessions growing in acid soils. Further research is needed to identify the P-uptake and P-utilization mechanisms by these accessions. Doing so will provide valuable information for wheat researchers interested in maximizing P-use efficiency.

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TABLES AND FIGURES

Table 2.1 Chemical characteristics of the Richfield clay loam (calcareous) and Lucien sandy loam (acid) Series soils.

Soil Series	pH	NO_3-N	Extractable P	Total P	Exch. Al	K	SO_4-S	Ca	Mg	Fe	Zn	B	Cu
			----------	----------	mg kg^{-1}	----------	----------	----------	----------	----------	----------	----------	----------
Richfield	7.9	1	8[§]	2131[¥]	0.1	564	7	4032	502	12.3	0.3	0.7	1.2
Lucien	4.8	19	6[±]	1906[¥]	0.3	106	19.5	673	214.5	30.3	0.9	0.2	1.0

§ Bicarbonate extractable P
± Mehlich 3 extractable P
¥ EPA 3050 Total P

Table 2.2 Plant mean biomass, phosphorus (P) uptake, and % P of plant tissue for accessions grown in low P acid soil in large pots (Pot_L) and small pots (Pot_S).

	Pot_L			Pot_S		
Accession	Biomass (mg)	P uptake (mg)	Plant P conc. (mg P g plant^{-1})	Biomass (mg)	P uptake (mg)	Plant P conc. (mg P g plant^{-1})
(1) TN801¥	274 a*	0.142	0.52	121 abcde	0.081	0.67
(2) P03207A-7¥	403 a	0.246	0.61	105 def	0.071	0.68
(3) W98008J1¥	225 bc	0.137	0.61	93 efg	0.060	0.65
(4) MO4*5109¥	306 a	0.168	0.55	107 cdef	0.063	0.59
(5) Jerry¥	258 ab	0.183	0.71	88 efg	0.049	0.55
(6) SD06069¥	180 bc	0.106	0.59	155 a	0.115	0.74
(7) SD06165¥	64 bc	0.024	0.38	65 gh	0.044	0.68
(8) TX05V5614¥	208 bc	0.160	0.77	45 h	0.035	0.77
(9) OK91G103 (Chisholm-T)±	163 bc	0.098	0.60	112 cdef	0.077	0.69
(10) OK91G107 (Chisholm-S)§	237 bc	0.163	0.69	95 efg	0.076	0.8
(11) OK91G105 (Century-T)±	180 bc	0.086	0.48	72 fgh	0.046	0.64
(12) OK91G108 (Century-S)§	113 bc	0.055	0.49	84 efgh	0.061	0.73

(13) Duster±	130 bc	0.083	0.64	115 bcdef	0.035	0.3
(14) OK07209§	120 bc	0.097	0.81	78 fgh	0.017	0.22
(15) Endurance±	215 bc	0.112	0.52	125 abcde	0.083	0.66
(16) Ruby Lee§	135 bc	0.089	0.66	135 abcd	0.111	0.82
(17) Karl 92§	216 bc	0.110	0.51	153 ab	0.095	0.62
(18) Jagger±	119 bc	0.101	0.85	115 abcdef	0.072	0.63
(19) Fuller§	162 bc	0.092	0.57	145 abc	0.088	0.61
(20) Garrison¥	129 bc	0.052	0.40	138 abcd	0.096	0.7
(21) OCW00S063S-1B¥	86 bc	0.044	0.51	118 abcde	0.083	0.71
(22) OK08328¥	146 bc	0.098	0.67	118 abcde	0.071	0.6

* Indicates significant difference between accessions in same pot size at p=0.10
¥ Field tolerant to acidity, no 4DL marker
± Tolerant to acidity, 4DL marker present
§ Susceptible to acidity

Table 2.3 Plant mean biomass, phosphorus (P) uptake, and %P of plant tissue for accessions grown in low P calcareous soil in large pots (Pot_L) and small pots (Pot_S).

	Pot_L			Pot_S		
Accession	Biomass (mg)	P uptake (mg)	Plant P conc. (mg P g plant^{-1})	Biomass (mg)	P uptake (mg)	Plant P conc. (mg P g plant^{-1})
(1) TN801¥	389 ab*	0.692	1.78	108 cdefgh	0.072	0.67
(2) P03207A-7¥	478 a	0.798	1.67	150 abcd	0.138	0.92
(3) W98008J1¥	279 bc	0.401	1.44	125 bcdef	0.096	0.77
(4) MO4*5109¥	489 a	0.792	1.62	145 abcde	0.090	0.62
(5) Jerry¥	282 bc	0.341	1.21	68 ghi	0.055	0.81
(6) SD06069¥	464 a	0.956	2.06	168 ab	0.141	0.84
(7) SD06165¥	416 ab	0.628	1.51	43 i	0.029	0.68
(8) TX05V5614¥	426 ab	0.755	1.77	63 hi	0.054	0.86
(9) OK91G103 (Chisholm-T)±	203 bc	0.470	2.32	84 fghi	0.062	0.74
(10) OK91G107 (Chisholm-S)§	335 ab	0.379	1.13	123 bcdefg	0.126	1.02
(11) OK91G105 (Century-T)±	402 ab	0.543	1.35	78 fghi	0.046	0.59
(12) OK91G108 (Century-S)§	385 ab	0.597	1.55	100 defgh	0.072	0.72

(13) Duster±	286 bc	0.171	0.60	120 bcdefg	0.084	0.7
(14) Iba§	359 ab	0.664	1.85	70 fghi	0.068	0.96
(15) Endurance±	323 bc	0.394	1.22	118 bcdefg	0.096	0.82
(16) Ruby Lee§	324 bc	0.538	1.66	180 a	0.130	0.72
(17) Karl 92§	403 ab	0.528	1.31	125 bcdef	0.081	0.65
(18) Jagger±	270 bc	0.460	1.70	155 abc	0.130	0.84
(19) Fuller§	270 bc	0.194	0.72	193 a	0.162	0.84
(20) Garrison¥	361 ab	0.595	1.65	118 bcdefg	0.085	0.72
(21) OCW00S063S-1B¥	314 bc	0.292	0.93	90 efghi	0.049	0.55
(22) OK08328¥	293 bc	0.489	1.67	160 abc	0.120	0.75

* Indicates significant difference between accessions in same pot size at p=0.05
¥ Field tolerant to acidity, no 4DL marker
± Tolerant to acidity, 4DL marker present
§ Susceptible to acidity

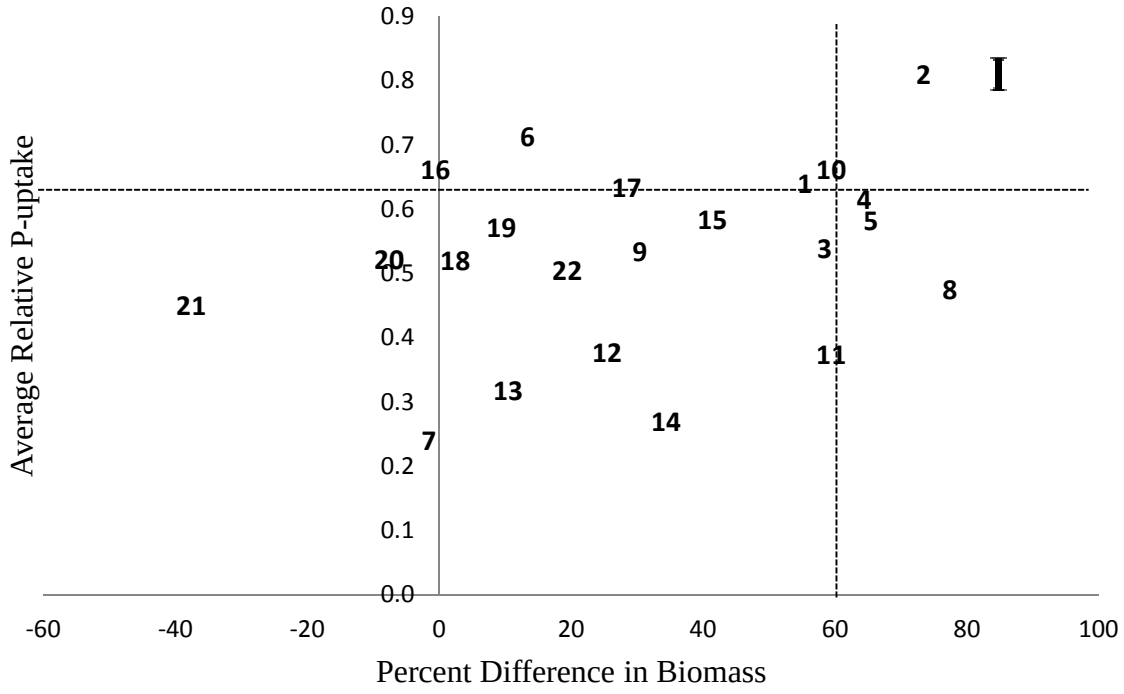

Figure 2.1 Phosphorus (P) uptake efficiency in acid soil. Numbers on the plot indicate accessions listed in Table 2. Vertical line indicates the 75th percentile for the percent difference in biomass between Pot_S and Pot_L (equation 3) and horizontal line indicates the 75th percentile for average (Pot_S and Pot_L) relative P uptake (equation 2). Accessions above the horizontal line display the highest P- uptake efficiency, while those in quadrant I are dominated with P-uptake efficiency with the least interference from P-utilization efficiency mechanisms.

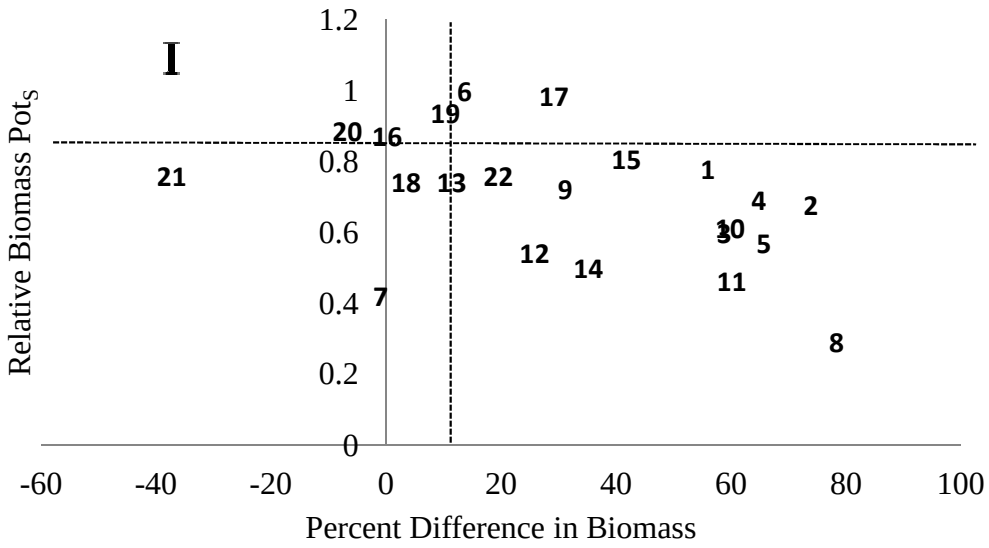

Figure 2.2 Phosphorus (P) utilization efficiency in the acid soil. Numbers in plot indicate acessions listed in Table 2. Vertical line indicates the 25th percentile for percent difference in biomass between Pot_S and Pot_L (equation 3) and the horizontal line indicates the 75th percentile for relative biomass for Pot_S (equation 4). Accessions located in quadrant I display the highest P-utilization efficiency.

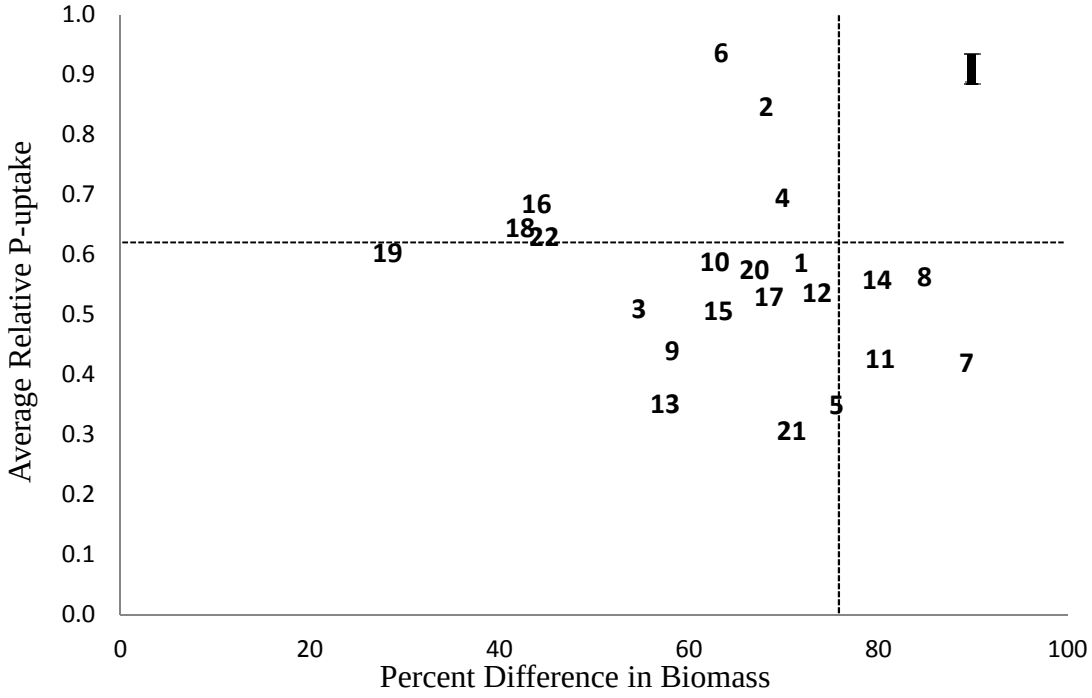

Figure 2.3 Phosphorus (P) uptake efficiency in calcareous soil. Numbers on the plot indicate accessions listed in Table 2. Vertical line indicates the 75th percentile for the percent difference in biomass between Pot_S and Pot_L (equation 3) and horizontal line indicates the 75th percentile for average (Pot_S and $Pot_{L)}$ relative P uptake (equation 2). Accessions above the horizontal line display the highest P- uptake efficiency, while those in quadrant I are dominated with P-uptake efficiency with the least interference from P-utilization efficiency mechanisms.

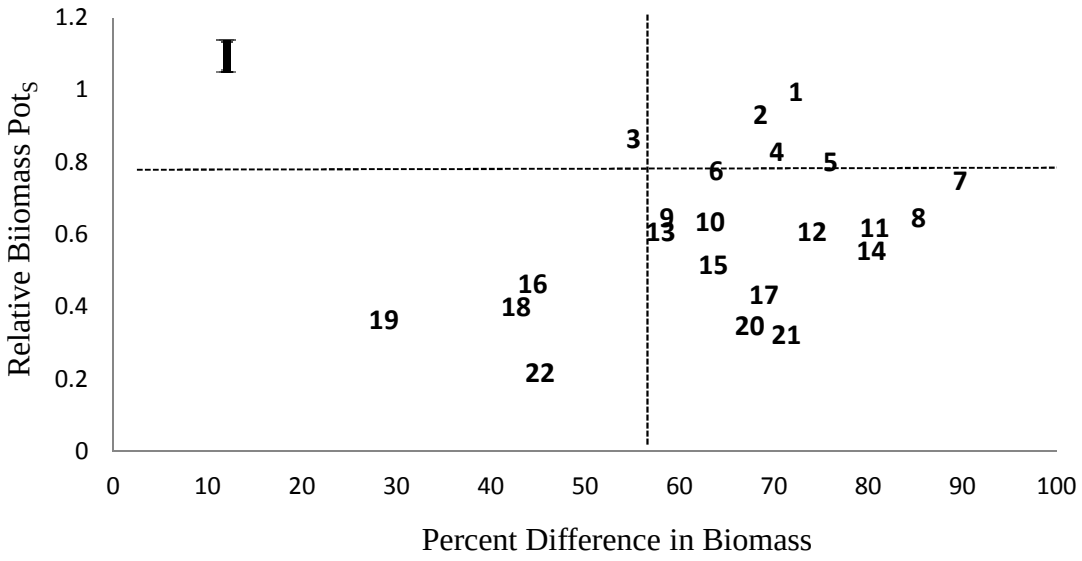

Figure 2.4 Phosphorus (P) utilization efficiency in the calcareous soil. Numbers in plot indicate accessions listed in Table 2. Vertical line indicates the 25th percentile for percent difference in biomass between Pot_S and Pot_L (equation 3) and the horizontal line indicates the 75th percentile for relative biomass for Pot_S (equation 4). Accessions located in quadrant I display the highest P-utilization efficiency.

CHAPTER III

INVESTIGATION OF PHOSPHORUS UPTAKE EFFICIENCY IN THE RHIZOSPHERE OF WHEAT GROWN IN ACID SOILS

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ABSTRACT

With the phosphate reserves predicted to be depleted in the next 40-50 years, it is imperative that phosphorus (P)-use efficiency be maximized to ensure global food production. The objective of this study was to evaluate the rhizosphere soil of three P-uptake efficient and three P-uptake inefficient accessions to determine plant mechanisms used to increase uptake of typically non-bioavailable P. The wheat plants were grown in rhizo-cells allowing for separate analysis of five separate soil layers distinct from the root surface. Soils were analyzed for organic acids, acid phosphatase enzymes, and phosphorus pools. Biomass and P-uptake for each accession were also measured. Overall, oxalic and citric acid, and pH changes were observed to be the most important contributors to increased P-uptake. Two pH sensitive pools composed of primarily Fe, Al, and Ca phosphates were the pools most affected by these plant exudates.

INTRODUCTION

Worldwide, phosphorus (P) is limiting on 30% of arable crop land (Vance et al., 2003; Korkmaz et al., 2009). Phosphorus is an essential macronutrient needed by plants as it is a vital component of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the cell nucleus (Troeh and Thompson, 1993b). Phosphorus also plays an essential role in respiration; hydrolysis of the terminal phosphate group in adenosine triphosphate (ATP) during its conversion to adenosine diphosphate (ADP) provides the energy needed for many plant processes (Noggle and Fritz, 1983). Thus, low plant P concentrations can disrupt physiological processes and result in yield loss. Phosphorus is a macronutrient and required by plants at an amount second only to nitrogen. To address P limitation, P fertilizer is applied in order to increase yields. A common alternative is to convert marginal lands into production in order to meet cereal demand when P fertilizers are not accessible or economically viable (Runge-Metzger, 1995). However, this expansion into marginal land can lead to increased rates of land degradation and further loss of soil fertility (Young, 2005). Additionally, the P reserves that society relies on to supply P fertilizers are decreasing in both quantity and quality and are projected to be depleted within the next 40-60 years (Stewart et al., 2005). Increased demand for P fertilizers and the decreasing supply has caused a tremendous increase in P fertilizer cost that will no doubt continue into the future with the price of super phosphate increasing from \$221 ton^{-1} in 2002 to \$665 ton^{-1} in 2012 (Steen, 1998; USDA, 2013). On top of this dire situation, global cereal demand is predicted to increase 31% to 2.83 billion metric tons by 2030 (Bruinsma, 2003; WOAB, 2011).Therefore, it is of upmost importance to increase the

ability of crops to produce high yields with less P input and also to utilize or convert organic wastes containing P into usable fertilizer.

Phosphorus efficiency

In non-agricultural ecosystems, plants often exhibit P use efficiency. Forest plants are able to produce large amounts of biomass in soils that contain very little typically labile P (Chen et al., 2003). It has also been noted that certain accessions of agricultural crops are more productive than others when grown in low P soils (Osborne and Rengel, 2002; Ozturk et al., 2005; Korkmaz et al., 2009). Phosphorus use efficiency in plants can be separated into two different strategies; P-utilization efficiency and P-uptake efficiency (Batten, 1992; Osborne and Rengel, 2002). P-utilization efficiency is a plant's ability to produce high biomass relative to the amount of phosphorus it has extracted from the soil. Plants achieve this through metabolic, morphological and physiological responses. This includes using alternative metabolic pathways and increasing root-shoot ratio (Hammond et al., 2004). Phosphorus-uptake efficiency involves uptake of a relatively large amount of soil P from a relatively non-labile P pool. (Föhse et al., 1991).

Phosphorus in soils can be categorized into three pools; solution P, active P, and fixed P. The solution P pool is usually in the orthophosphate form (HPO_4^{2-} and $H_2PO_4^-$), making it readily available for plant uptake. However, this pool is very small compared to the total P in the soil. The active P pool is composed of readily soluble inorganic P and easily mineralized organic P. The active P pool releases P into solution as the solution P pool decreases. The fixed P pool consists of relatively insoluble inorganic P that releases P into the active pool, although not in a large quantity (Busman et al., 2002). Soil P is

found in three general chemical forms in the soil; organic P, calcium (Ca)-bound inorganic P, and iron (Fe)- or aluminum (Al)-bound inorganic P (Brady and Weil, 2008). Organic P forms include phospholipids, nucleic acids, and inositol phosphates (Tisdale and Nelson, 1975). The fraction of soil organic P is small compared to inorganic P for most soils.

Soil pH plays a major role in the form of inorganic P found in soils. Iron- or aluminum-bound P is found predominately in acid soils. Phosphorus can also be fixed to soil particles through interaction with valence-unsatisfied edges on soil minerals. In this reaction, phosphate ions either replace a hydroxyl group from an aluminol or form a clay-Ca-phosphate linkage (Tisdale and Nelson, 1975). The ability of a soil to fix P changes with varying amounts and types of clay minerals, organic matter, and variation in soil pH (Brady and Weil, 2008).

To achieve P-uptake efficiency, plants exude various compounds such as organic acids and phosphatase enzymes into the soil that make typically non-labile P pools more available for uptake by the plant or by adjusting the rhizosphere soil pH (Hedley et al., 1982; Tarafdar, 1987; Dinkelaker et al., 1989; Hocking, 2001).The organic acids exuded by plants vary between species and between accessions and include oxalic acid, citric acid, and malic acid. The organic acids can either chelate metal ions that are bound with P or compete with phosphate ions for adsorption sites on soil colloids thus freeing up P into solution, making it more accessible by plants (Hocking, 2001). Phosphatase enzymes remove phosphate groups from phytic acid (the most common form of soil organic P), rendering it plant available. Phosphatase enzyme excretion can be especially important considering the large amounts of inorganic P fertilizer that is immobilized by

microorganisms into organic P (Richardson et al., 1994). Soil pH modification occurs when plants excrete H^+ or HCO_3^- when cation/anion uptake is not in balance (Grinsted et al., 2011). Proton excretions cause a decrease in rhizosphere pH, causing acid-soluble forms of inorganic P to become available through dissolution (Hedley et al., 1982). In addition to the exudation of these compounds, plants can also increase P-uptake by improved root architecture. Through this strategy, the roots have much more surface area through a higher density of root and root hair formation. This increased surface area increases the amount of soil the roots are in contact with and thus increases the possibility of extracting P from soils.

Additionally, when P fertilizer is applied, only 10-20% of that applied is recovered during the growing season (Sharpley, 1986; Mclaughlin et al., 1988). This happens because added phosphorus converts into forms that are typically not accessible by plants. Phosphorus-uptake efficient plants can more effectively uptake this applied P by accessing the non-labile forms of P formed, thus increasing efficiency.

Of the 17.8 million metric tons P_2O_5 applied as fertilizer globally, wheat uses 16.4%; second only to high value fruit and vegetable crops (Heffer, 2009).Therefore, increasing P-use efficiency in wheat can lead to great gains in meeting the increased cereal demand while decreasing the negative environmental and social consequences evident with P inefficiency. If producers can apply less P while still achieving the same yields, this can decrease potential P losses in runoff to surface waters, which leads to eutrophication. Studies have often focused on P-utilization efficiency (Gardiner and Christensen, 1990; Föhse et al., 1991; Bhadoria et al., 2002; Manske et al., 2002). While

these studies are indelibly useful, much work is still needed to examine the role of root exudates in the P-uptake efficiency of wheat.

In order to increase the P-uptake efficiency of wheat, studies investigating various root exudates and their impact on the many types of P found in soils are warranted. These mechanisms need to be identified and further studied for various accessions as they typically differ between different lines (Hocking, 2001).This information will provide plant breeders with information that will help to focus their program's efforts when working to increase P-uptake efficiency in their wheat lines.

METHODS

Twenty-two wheat accessions were screened in a low P acid soil in a previous experiment to distinguish which accessions demonstrated mostly P-uptake or P-utilization efficiency. The three most P-uptake efficient and three most P-uptake inefficient accessions were chosen for this experiment. Accessions were grown in rhizo-cells, modified from Hedley et al. (1982), with 0.25g of wheat seed per pot. The rhizo-cells are designed so that plant roots can interact with the soil but cannot grow into the soil, eliminating the effect of root growth and architecture as a possible P-uptake strategy. The rhizo-cells consisted of two, five cm diameter columns connected to each other by adhering them together with tape. Each compartment was 7.62 cm tall and separated by a 0.30 μm mesh screen. The bottom compartment consisted of low-P acid soil. The top compartment contained the seeds, which were placed directly on the 0.30 μm mesh screen, and covered with 0.5 inches of pure lab-grade sand (Acros Organics, 40-100mesh) to cover the seeds.

Two sets of rhizo-cells were established in order to measure root excretions and changes in soil pH and P pools; the first set (four replications) was used to measure soil pH, phosphatase, and P fractionations, and the second (three replications) to measure organic acids. Rhizo-cells were placed in a growth chamber and plants grown for twenty-eight days after emergence. Daytime temperatures were 25 ^{o}C and nighttime temperatures were 20 ^{o}C. The plants received 16 hours of daylight at a lighting intensity of 200 µmoles m^{-2} per s. Each Rhizo-cell was placed in a water reservoir containing a 1400 mg L^{-1} potassium nitrate (KNO_3) fertilizer solution. This allowed the soil to remain near constant field capacity while ensuring that the plants did not experience nitrogen deficiency. The soil contained adequate K and micronutrients (Table 3.1).

Prior to the experiment, the soil was dried at 65° C, sieved to 2 mm, and analyzed for pH, NO_3-N, K, SO_4-S, Ca, Mg, Fe, Zn, B, Cu, Total P, and extractable P and Al (Table 3.1). Soil pH was measured with a 1:1 soil to water suspension with a combination electrode (Thomas, 1996). Extractable P, K, Mg, and Ca were measured according to the method developed by Mehlich (1984) in which 2.0 g sample of soil is extracted with Mehlic 3 (M3) solution (0.015 *M* NH_4F, 0.2 *M* CH_3COOH, 0.25 *M* NH_4NO_3, 0.013 *M* HNO_3, 0.001 *M* EDTA) and shaken for 5 minutes. The solution was then filtered through a #2 Whatman filter paper and analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Spectro Ciros, Mahwah, NJ). For total P determination, 0.5 g of soil was subjected to an acid digestion according to the EPA 3050 method (EPA, 1986). Extracts from this procedure were then analyzed by ICP-AES. For nitrate nitrogen (NO_3-N), 2.5 g of soil was extracted with 10 mL of 1.0M KCl and shaken for 15 minutes. This solution was then analyzed for NO_3-N using Lachat Quickchem 8000 automated flow-

injection analyzer (Mulvaney, 1996). SO_4-S was measured by shaking 10 g of soil with 25 mL 0.008 *M* calcium phosphate, and subsequently analyzing with ICP-AES. (Schulte and Eik, 1988). Boron, Zn, Fe, and Cu were extracted by shaking 10 g of soil sample with 20 mL of DTPA- Sorbitol (0.005 *M* DTPA, 0.1 *M* triethanolamine (TEA), 0.01 *M* $CaCl_2$, and sorbital) for two hours. The extract from this was then analyzed by ICP-AES (Lindsay and Norvell, 1978). Extractable Aluminum was determined by shaking 5 g of soil sample and 20 mL 1.0 *M* KCl for 30 minutes, filtering through a #2 Whatman filter paper, and analyzing by ICP-AES (Page et al., 1982). Soil texture was determined using the hydrometer method (Gee and Baude, 1986).

Plant Analysis

After 28 days of growth, roots and shoots were harvested separately and dried for 24 hours at 85°C to determine plant biomass yield. Due to low biomass, replicates of roots and replicates of shoots were compiled together for each experiment, respectively. The composited samples were then digested (Jones and Case, 1990) and subsequently analyzed for P content by ICP-AES. The mass of P uptake was calculated using P concentration (mg P kg biomass $^{-1}$) and total biomsass (kg):

(1) $$P\ uptake\ (mg) = \frac{Plant\ P\ concentration \times total\ plant\ biomass}{number\ of\ plants\ in\ composite}$$

Soil Sampling

After harvesting plants from the rhizo-cells, soils were sliced at different depths for chemical analysis. For the first experiment the upper 3.75 mm of soil in the bottom compartment (under the mesh screen) was sliced into five 0.75 mm increments while the

second experiment was sliced into two 2.25 mm slices. To do so, Rhizo-cells were placed horizontally and the soil pushed uniformly from the bottom for the designated depth. The advanced soil was then sliced with a thin wire.

Soil Analysis

For the phosphatase assay, soil was analyzed for acid phosphatase according to the phosphatase assay method developed by Tabatabai (Tabatabai, 1994). For each depth, 0.2g of soil was collected without drying and placed in a small vial. To the soil, 0.2 mL of toluene, 4mL of modified universal buffer, and 1 mL p-nitrophenyl phosphate solution were added and the vial was stopped with a rubber stopper. The solution was then incubated for 1 hour at 37^0 C. After the incubation period, 1mL of 0.5 M $CaCl_2$ and 4mL of 0.5 M NaOH were added to the solution. The contents were gently swirled and then filtered through a no. 2v Whatman filter paper. The resulting solution was then measured for color intensity using a spectrophotometer set at a wavelength of 400 nm (Milton Roy Spectronic 21D). Standards were prepared by incubating various 0, 10, 20, 40, and 40 ug of p-nitrophenol according to the same method for the soil samples. However, the addition of 1 mL p-nitrophenyl phosphate was carried out after the solution had been incubated instead of before incubation. The measured results were then fit into a standard curve to determine the amount of *p*-nitrophenol released. Controls for each soil used were prepared and the results subtracted from the experimental samples. For this method, phosphatase activity is determined by the amount of *p*-nitrophenol released in the soil. This is expressed as the μmol *p*-nitrophenol g^{-1} soil per minute.

Soil pH was measured with a combination electrode in a 1:1 soil to water suspension (Thomas, 1996). Soil fractions were determined using the fractionation method developed by Hedley et. al (Hedley et al., 1982) with a slight modification. For each depth, 0.5 grams was used. Inorganic P was determined using a spectrophotometer according to the method developed by Murphy and Riley (Murphy and Riley, 1962) and total P was measured by complete digestion according to Tiesson and Moir (Tiessen and Moir, 1993) in which the solution was then also subjected to Murphy Riley analysis. Organic P was determined by subtracting the inorganic P from the total P measured. To determine solution P, the soil was shaken with 30 mL of DI H_2O and 1 resin strip (ResinTech, AMB-SS Anion Single Sheet Ion Exchange Membrane) overnight. The resin strip was then rinsed with DI H_2O and placed in 20 mL of 0.5 *M* HCl and shaken overnight. The resin strip was then removed and an aliquot of the solution was analyzed for inorganic phosphorus by spectrophotometer via the Murphey Riley method. The soil solution was centrifuged at 10,000 rpm for 15 minutes and the supernatant was decanted and thrown away. To determine the "loosely bound" P, 30 mL 0f 0.5 *M* $NaHCO_3$ was added to the soil and shaken overnight. The solution was then centrifuged and the supernatant was decanted and analyzed for both inorganic and organic P. For the next step, the soil was mixed with 30 mL 0f 0.1 *M* NaOH and shaken overnight. The solution was then centrifuged and the supernatant decanted and analyzed for inorganic and organic P. The pool represented by the extraction is predominantly Fe and Al-related phosphate, which includes P bound to Al and Fe hydroxide minerals. The soil was then extracted with 30 mL of 1 *M* HCl and shaken overnight. The solution was centrifuged, supernatant decanted, and analyzed for inorganic P. This pool represents primarily Ca

phosphate compounds. For the final step, the soil was subjected to a complete digestion to determine residual P (EPA, 1986). For this complete digestion, the soil was heated with concentrated HNO_3, concentrated HCl, and 30% H_2O_2.

The two depths of soil (Depth 1 [0-2.25 mm], Depth 2 [2.25-3.75 mm] from the second set of rhizo-cells were analyzed for organic acids. To do so, 5 g of each soil was shaken for two hours with 50mL of 0.01 *M* NaOH and then centrifuged at 20,000 rpm for 20 minutes. The supernatant was then filtered through a 0.45 µm filter using a vacuum pump. This solution was then subjected to purification through 5.0 of Amberlite (Amberlite IR120, H^+ form, Fluka Analytical) cation exchange resin. Solution was reacted with Amerblite resin with the aid of a vacuum pump, followed by rinsing with DI H_2O. The cation free solution was then reacted with 2.0 g of Dowex anion-exchange resin (Dowex 1x8 ion exchange resig, Sigma-Aldrich) and rinsed with DI H_2O. The Dowex resin was then washed with 8 mL of 1M HCl to release the organic acids into solution. This purified solution was then concentrated in a speed-vac (Savant Instruments, Holbrook, NY) and reconstituted with 1mL 0.05 *M* H_2SO_4 and then analyzed for oxalic, malic, and citric acids with high performance liquid chromatography (HPLC, Dionex 4500i; Sunnyvale, CA, USA) with VDM-2 variable wavelength detector, GPM-gradient pump, and AS3500 autosampler. The HPLC was outfitted with an Aminex HPX-87H column (Bio-Rad, Hercules, CA), guard column (Bio-Rad, Hercules, CA), and column heater (Eppendorf CH500, Hamburg, Germany) set at 60°C. The HPLC mobile phase was 0.005 M H_2SO_4 flowing at 0.6 ml min^{-1}. Organic acids were analyzed with a wavelength of 214 nm. Peak area of the three organic acids were analyzed using PeakNet software (Version 5.21; Dionex).

Statistical Analysis

All data were analyzed using SAS software package (SAS Institute, 2009). Duncan Groupings between all accessions and within pools and depths were computed using means statements with the GLM procedure with an alpha value of 0.05 being significant. The Stepwise Regression model for P-uptake was executed using the REG procedure and independent variables were kept if significant at an alpha value of 0.10. Single Pearson Correlation coefficients were computed using the CORR procedure for single correlations between P-uptake and the phosphorus pools, exudates, and biomass for two depths (Table 3.4).

RESULTS AND DISCUSSION

Plant growth and phosphorus uptake

Accessions 4, 10, and 2 were characterized from a previous study to be P-uptake efficient while accessions 7, 13, and 14 were characterized as being P-uptake inefficient. For this study, accession 4 took up 0.62 mg of P and accession 10 took up 0.64 g of P (Table 3.2). This is almost twice as much P than was taken up by the other cultivars. While accession 2 was characterized as being P-uptake efficient in the previous study, it appears that it exhibits predominantly P-utilization efficiency as it only took up 0.30 mg of P but still yielded 500.8 mg of biomass, making it the third highest biomass producer. However, the other accessions appear to have been characterized correctly based on their P-uptake ability as biomass showed a significant positive relationship with P-uptake (Table 3.4).

Although the soil was acidic, there were very low levels of exchangeable aluminum (Al), making it a non-Al toxic acid soil (Figure 3.1). For this reason, the study focuses only on P-uptake efficiency mechanisms without the confounding influence of Al-toxicity. This can be observed as accession 10 is susceptible to Al-toxicity but is found to be very efficient at P-uptake and therefore biomass production.

Chemical mechanisms in the soil

The multiple linear regression (i.e. step-wise regression) was conducted with P-uptake as the dependent variable and soil phosphatase enzyme, organic acid concentrations, pH, and the various P-fractions as the independent variables. The multiple linear regression was executed for all depths combined, for only depth 1 (0-2.25 mm), and for only depth 2 (2.25-3.75 mm). Single correlations were also conducted. A negative correlation coefficient (from either single correlations or multiple linear regression) between a soil P-form and P uptake indicated that the specific P form was accessible and taken up by the plant.

For all depths combined, oxalic and citric acids were retained as significantly ($p=0.10$) contributing to P-uptake with oxalic having a partial R^2 of 0.75 and citric acid of 0.20. The P-uptake efficient accessions had the highest organic acid exudation overall (Table 3.5). This exudation of organic acids could be contributing to P-uptake efficiency by chelating Fe and Al in Fe and Al phosphate complexes, thus releasing phosphate into solution (Hocking, 2001). This has been observed in many plant species (Grierson, 1992; Hocking, 2001), but is highly variable between accessions within species as organic acid exudation is highly stress and plant-species specific (Jones, 1998). This mechanism is

especially important as acid soils are typically dominated with P in the form of Al and Fe-bound P (i.e. NaOH extractable P; Table 3.3). There was little variation between accessions in terms of the phosphatase enzymes exuded (Table 3.5).

With regard to inorganic NaOH extractable P (NaOH-IP), organic NaOH extractable P (NaOH-OP), and $NaHCO_3$-IP, there is a distinct difference in the first three depths nearest the roots and the last two (Figures 3.1, 3.2, and 3.3). This reaffirms the idea that the rhizosphere is approximately 2.0 mm of soil surrounding the roots (Pinton et al., 2001). For this reason, stepwise regression models were executed with the top three depths compiled as the rhizosphere and the bottom two depths were compiled as the bulk soil. Notice that for inorganic NaOH-P (i.e. Fe and Al-bound P), accession 10 displayed an increasing trend with distance from the root surface (Figure 3.1; Table 3.1). This is likely due to the fact that this accession excreted the highest concentration of oxalic acid, which solublizes Al and Fe-bound P. A similar trend is observed for organic NaOH-P (Figure 3.3) as accession 4 seems to access this P pool through both organic acids, which help to make Al and Fe-phytate more available to phosphatase enzymes. Accession 4 excreted the highest total organic acids (sum of oxalic, citric, and malic) and among the highest for phosphatase enzyme excretion at 0-2.25 mm from the root surface. Similarly, accession 4 also appeared to be able to access the inorganic $NaHCO_3$-P pool (Figure 3.2).

Rhizosphere Soil (depth 1)

For the rhizosphere both citric and oxalic acid were retained as significant variables with a partial R^2 of 0.78 and 0.15, respectively. In addition to these two organic acids, pH (partial R^2=0.01) and HCl-P (partial R^2=0.06) were retained as significant

variables. While the regression model does not calculate indirect effects, it can be inferred from the stepwise regression results that there is an indirect effect of pH on the HCl-P fraction. In other words, at lower pH levels, there is less HCl-P found in depth 1 for all accessions combined. By the very nature of the HCl-P fraction being the "acid soluble" P fraction, it can be inferred that lower pH levels dissolve this fraction and subsequently make it more accessible to plants (Hedley et al., 1994). The P-uptake efficient accessions on average had lower pH levels in the rhizosphere than non-efficient accessions (Table 3.3). Part of this pH change is attributed to the general exudation of protons (H^+) from the plants functioning to maintain charge balance as they uptake cations (Taiz and Zeiger, 2010; Grinsted et al., 2011). However, Hedley et al. (1994) suggests that pH changes alone are not responsible for all P solubilization; instead he hypothesizes that various low molecular weight organic acids are contributing to this phenomena. The present study supports that hypothesis as organic acids were also determined to be significant variables in regard to P-uptake.

In regard to single correlation coefficients, oxalic acid had a significant correlation with P-uptake at a R^2=0.86 (Table 3.4). Accessions 10 and 4 had both the highest amount of oxalic acid in this depth and the highest P-uptake overall. On the other hand, accession 2 had the lowest oxalic acid exudation and exhibited nearly the lowest amount of P uptake (Table 3.5). In addition to oxalic acid, citric acid was identified by the stepwise regression model as a significant factor for P-uptake. While soil concentrations of citric acid were relatively low for accessions that did exude them, virtually none of the P-uptake inefficient accessions exuded any detectable citric acid (Table 3.5). These two organic acids were found to be significant in making various

forms of P available in the rhizosphere of maize in a study conducted by Bolan et al. (1994).

Known for their metal-complexing ability, these organic acids can chelate metals in metal-phosphorus compounds, thus releasing phosphate into solution. Parker et al. (2005) stated that under normal rhizosphere conditions, oxalic and citric acid have the ability to significantly alter metal speciation, and thus P chemistry in the soil. Under normal rhizosphere conditions, these two ligands have complex formation constants with Ca^{2+} of $\log K_f$ =4.8 for citrate and $\log K_f$ = 3.0 for oxalate (Essington, 2004). The large complex formation constants with Ca^{2+} indicate that even at low concentrations, citric and oxalic acid have a great ability to complex with Ca^{2+}. This is consistent with the results of this study, which suggests that citric acid is still involved in P-uptake even at low concentrations. In addition to chelation of metals, organic acids contribute to soil acidification by deprotonization depending on each organic acids K_a constant (Liu, 2004). Both oxalic and citric acid are diprotic with pK_a values of 1.12 and 4.19 for oxalic acid and 3.14 and 4.77 for citric acid (Perrin et al., 1981), which means that they donate a proton to the solution if the pH value is greater than their pKa value.

Bulk Soil (depth 2)

The multiple linear regression model identified pH (partial R^2=0.72), $NaHCO_3$-IP (partial R^2=0.23), HCl-P (partial R^2=0.05), and NaOH-IP (partial R^2=0.0008) as significantly affecting P-uptake (Table 3.6). While oxalic acid was not retained as significantly contributing to the stepwise regression model, it was found to be significant by the single Pearson correlation test with P-uptake at depth 2 (R^2=0.85, Table 3.4). This

suggests that some accessions have the potential to impact solution chemistry at a distance greater than 2.25 mm from the root surface. The higher oxalic acid concentrations excreted by the P-uptake efficient accessions compared to non-efficient accessions at the greater depth suggest that oxalic acid could have an indirect effect on the P fractions chosen by the regression model. In addition to the HCl-P being sensitive to dissolution by acids, the $NaHCO_3$-IP fraction is viewed as being one of the more plant accessible fractions because it is relatively loosely held P that is sensitive to pH changes (Hedley et al., 1994); this pool is typically composed of a mixture of Al, Fe, and Ca-related P. Oxalic acid may have had an impact on the availability of this P pool.

Because the NaOH-IP fraction generally represents Fe and Al-bound phosphates, the lower pH for P-uptake efficient accessions does not explain why NaOH-IP plays a significant role in the stepwise regression model. However, the presence of oxalic acid does affect the NaOH-IP. Oxalic acid has the following stability constants with Fe and Al; Fe(II)=>4.7, Fe(III)=9.4, Al(III)=7.26 (Furia, 1972). These relatively high stability constants indicate that Oxalic acid has a strong tendency to chelate with these metals, which typically are in the form of Al-, and Fe-phosphates at this pH, thus freeing up phosphate into solutions and making it more accessible for plant uptake.

CONCLUSION

This study indicates that low molecular weight organic acids, particularly oxalic and citric acid play important roles in P-uptake ability for the accessions screened. In addition to these organic acids, accessions that were able to maintain an acidic rhizosphere were better able to uptake typically recalcitrant forms of P; this may simply

be a secondary effect of the excreted organic acids. From the stepwise regression, we gathered that the P-uptake efficient accessions are better able to access the typically non-plant available HCl-P and NaOH-IP fractions, as well as the $NaHCO_3$-IP pool. The study also concludes that accessions remain highly variable in their ability and means of acquiring P from the soil. However, these differences should be studied for tradeoffs in regard to energy of producing and exuding the above mentioned plant exudates and the benefit incurred from the increased P-uptake.

It is also prevalent in this study that while the rhizosphere remains very important for nutrient acquisition, studies should not be limited to this area as plant accessions still were able to access and influence the bulk soils outside of the rhizosphere. Additionally, this study reaffirms that selection criteria from the previous experiment for pinpointing accessions with P-uptake efficiency are both valid and useful.

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TABLES

AND FIGURES

Table 3.1 Chemical characteristics for Lucien sandy loam (acid) Series soils.

Soil Series	pH	NO_3-N	Extractable P	Total P	Exch. Al	K	SO_4-S	Ca	Mg	Fe	Zn	B	Cu
			----------mg kg^{-1}----------										
Lucien	4.8	19	6[±]	1906[¥]	0.3	106	19.5	673	214.5	30.3	0.9	0.2	1.0

§ Bicarbonate extractable P
± Mehlich 3 extractable P
¥ EPA 3050 Total P

Table 3.2 Plant mean biomass and phosphorus (P) uptake for each accession.

Accession	Mean Biomass (mg)	P-uptake (mg)
(4) MO4*5109¥	728.3a	0.62
(10) OK91G107 (Chisholm-S)§	696.4a	0.64
(2) P03207A-7¥	500.8a	0.30
(7) SD06165¥	429.3a	0.34
(14) Iba§	414.4a	0.32
(13) Duster±	383.5a	0.30

¥ Field tolerant to acidity, no 4DL marker
± Tolerant to acidity, 4DL marker present
§ Susceptible to acidity

Table 3.3 Soil phosphorus (P) forms and pH after 28 days of wheat growth in rhizo-cells. Values are average of 0-2.25 mm (depth 1) and 2.25-3.75 mm (depth 2) for each accession.

		Inorganic Phosphorus				Organic Phosphorus			
		Resin	0.5 *M* $NaHCO_3$	0.1 *M* NaOH	1.0 *M* HCl	0.5 *M* $NaHCO_3$	0.1 *M* NaOH	EPA 3050 Digestion	pH
Accession	Depth	----------mg P kg^{-1} soil----------							
(4) MO4*5109	1	1.04a	4.79a	22.9a	4.97a	0.67a	471.98b	1611.7a	4.82a
	2	0.99a	5.67a	22.84a	4.61a	0a	575.06a	1540.61a	4.42a
(10) OK91G107	1	1.05a	6.5a	22.19a	5.6a	0a	901.44a	1342.24a	4.48a
	2	0.9a	4.73a	23.04a	4.73a	0.04a	746.72a	1287.22a	4.53a
(2) P03207A-7	1	0.97a	6.17a	21.54a	5.74a	1.97a	763.43ab	1128.59a	5.24a
	2	1.24a	5.32a	21.97a	4.85a	0.02a	772.41a	1136.05a	4.99a
(7) SD06165	1	1.02a	5.85a	19.44a	4.61a	0a	765.8ab	818.37a	5.21a
	2	1.92a	4.78a	19.83a	4.73a	0a	765.79a	833.14a	5.07a
(14) Iba	1	1.22a	6.87a	21.7a	5.18a	0.22a	757.1ab	1085.76a	4.76a

	2	1.27a	6.88a	24.96a	4.96a	0a	740.97a	1102.09a	4.67a
(13) Duster	1	2.57a	3.79a	18.81a	5.56a	0.73a	474.77b	1582.39a	4.96a
	2	1.35a	4.2a	21.22a	6.82a	0.12a	536.18a	1504.59a	5.05a

Data with the same lower case letter indicates no significant difference between the same P-fraction of other accessions for depth 1 (p=0.05). Data with same upper case letter indicates no significant difference between the same P-fraction of other accessions for depth 2 (p=0.05).

Table 3.4 Correlation Coefficients between phosphorus (P) uptake and soil P pools, exudates, and biomass determined from rhizo-cells after 28 days of wheat growth. Phosphorus pools, pH, and exudates correlated were the average of 0-2.25 mm (depth 1) and 2.25-3.75 mm (depth 2).

	Inorganic Phosphorus				Organic Phosphorus								
Depth	Resin	0.5 *M* $NaHCO_3$	0.1 *M* NaOH	1.0 *M* HCl	0.5 *M* $NaHCO_3$	0.1 *M* NaOH	EPA 3050 Digestion	pH	Phosphatase Enzyme	Oxalic Acid	Citric Acid	Malic Acid	Biomass
1	-0.38	0.05	0.69	-0.04	-0.34	0.05	0.47	-0.52	0.53	0.86*	-0.27	-0.18	0.86*
2	-0.67	-0.03	0.27	-0.46	-0.22	-0.15	0.45	-0.85*	0.08	0.85*	0.60	0.79*	

- Indicates significances at p=0.05

Table 3.5 Soil concentrations of phosphatase enzyme, oxalic acid, citric acid, and malic acid extracted from rhizo-cells after 28 days of wheat growth. Values shown are the average of 0-2.25 mm (depth 1) and 2.25-3.75 mm (depth 2) for each accession.

Accession	Depth	Phosphatase Enzyme (μmol *p*-nitrophenol g^{-1} soil/ min)	Oxalic Acid (μmol g^{-1} soil)	Citric Acid (μmol g^{-1} soil)	Malic Acid (μmol g^{-1} soil)
(4) MO4*5109	1	0.07325a	0.07601a	0.0a	0.05515a
	2	0.05969A	0.07218A	0.002303A	0.04981A
(10) OK91G107	1	0.07533a	0.08555a	0.00330a	0.03898a
	2	0.07819A	0.07821A	0.0A	0.06844A
(2) P03207A-7	1	0.05615a	0.02276a	0.02521a	0.05741a
	2	0.06194A	0.04184A	0.0A	0.04772A
(7) SD06165	1	0.04963a	0.04811a	0.0a	0.03221a
	2	0.04815A	0.05985A	0.0A	0.04108A
(14) Iba	1	0.07796a	0.05322a	0.0a	0.05025a
	2	0.07746A	0.06372A	0.0A	0.03927A
(13) Duster	1	0.06408a	0.05538a	0.0a	0.05675a
	2	0.06627A	0.05038A	0.0A	0.04275A

Data with the same lower case letter indicates no significant difference between the same P-fraction of other accessions for depth 1 (p=0.05). Data with same upper case letter indicates no significant difference between the same P-fraction of other accessions for depth 2 (p=0.05).

Table 3.6 Stepwise regression model for estimation of phosphorus (P) uptake by wheat after 28 days of growth in rhizo-cells. Soil P pools, pH, and exudates used in the model were the average of 0-2.25 mm (depth 1) and 2.25-3.75 mm (depth 2). All variables retained for the model were significant at p=0.10

	Variable	Parameter Estimate	Partial R^2	Model R^2
All Depths	Intercept	-0.31	--	--
	Oxalic Acid	11.60	0.75	0.75
	Citric Acid	18.74	0.20	0.95
Depth 1 (Rhizosphere)	Intercept	1.36	--	--
	Oxalic Acid	9.5	0.78	0.78
	Citric Acid	14.95	0.15	0.93
	HCl-P	-0.15	0.06	0.99
	pH	-0.15	0.01	1.0
Depth 2 (Bulk Soil)	Intercept	4.18	--	--
	pH	-0.59	0.72	0.72
	$NaHCO_3$-IP	-0.10	0.23	0.95
	HCl-P	-0.05	0.05	1.0
	NaOH-IP	-0.007	0.0008	1.0

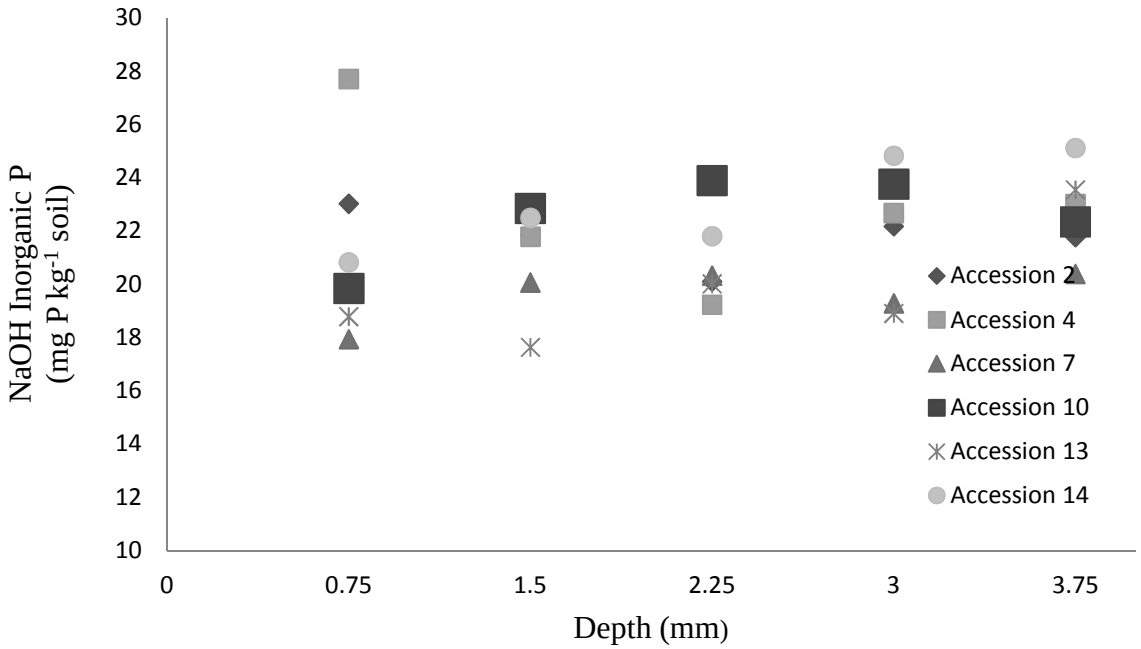

Figure 3.1 Changes in soil NaOH extractable inorganic phosphorus (P) with depth for each accession after 28 days of wheat growth in rhizo-cells.

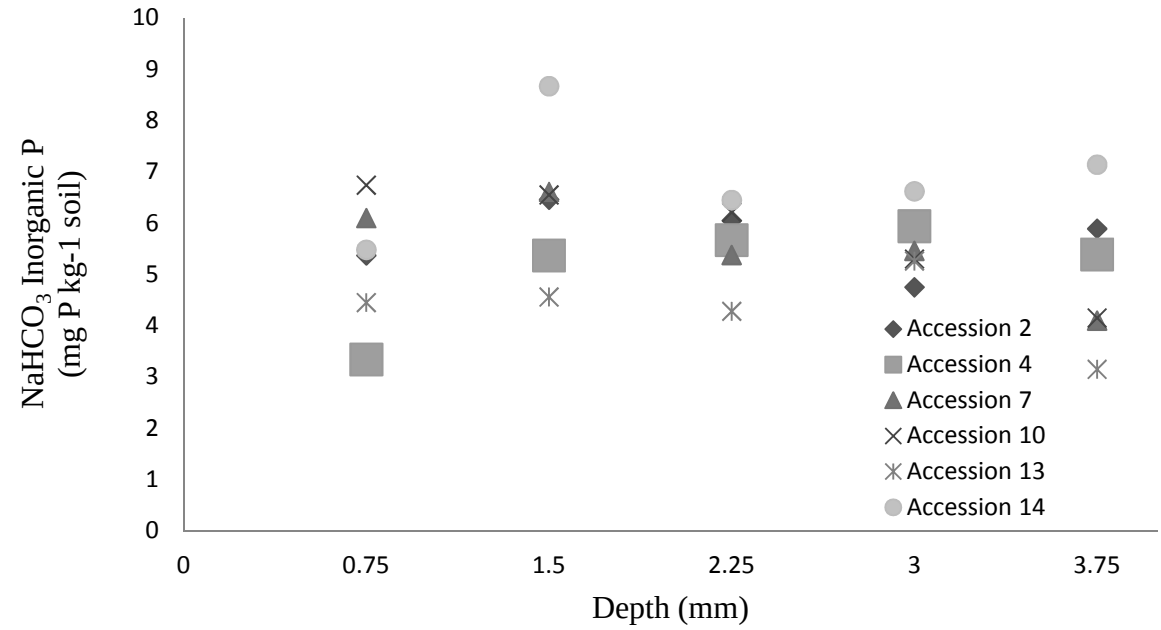

Figure 3.2 Changes in soil $NaHCO_3$ extractable inorganic phosphorus (P) with depth for each accession after 28 days of wheat growth in rhizo-cells.

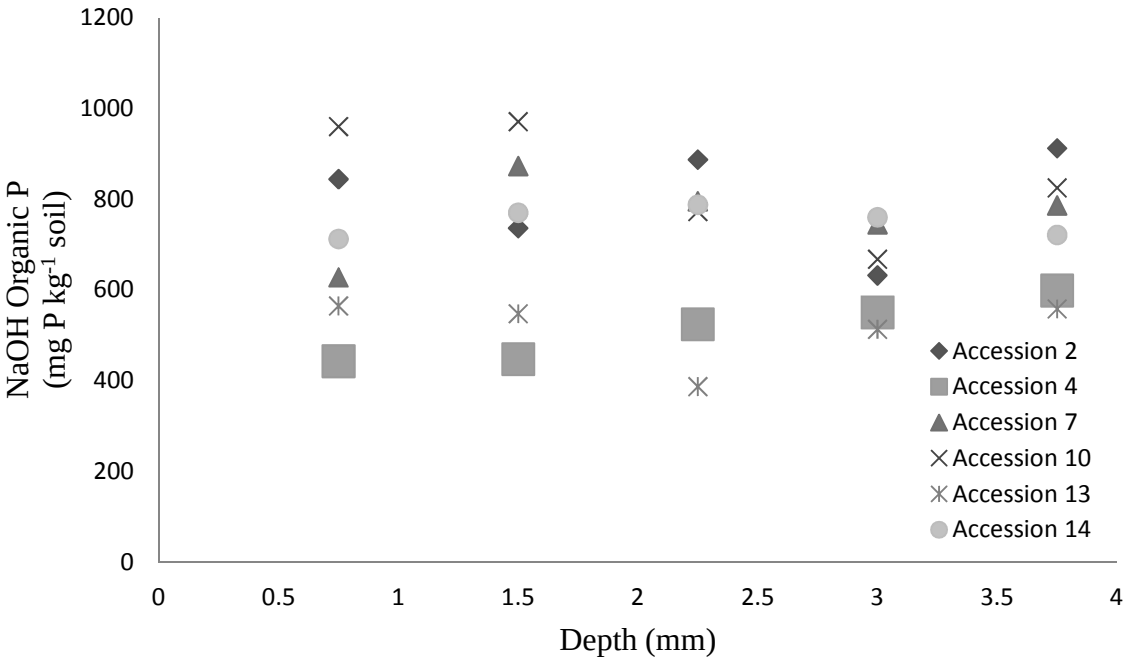

Figure 3.3 Changes in soil NaOH extractable organic phosphorus (P) with depth for each accession after 28 days of wheat growth in rhizo-cells.

CHAPTER IV

INVESTIGATION OF PHOSPHURS UPTAKE EFFICIENCY IN THE RHIZOSPHERE OF WHEAT GROWN IN CALCAREOUS SOILS

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ABSTRACT

In order to meet the growing demand for food in the midst of declining natural resources such as phosphorus (P), it is of upmost importance that our agricultural systems become more efficient in the use of nutrients. This study aimed to identify mechanisms employed by wheat plants in calcareous low P soils in order to uptake typically non-labile P. Three P-uptake efficient and three P-uptake inefficient accessions were grown in rhizo-cells for 28 days in a growth chamber. Soil layers distinct from the root surface were sampled at increasing distance away from the roots. Soils were analyzed for organic acids, alkaline phosphatase enzymes, and P forms. Plants were observed to be accessing the $NaHCO_3$-IP pool, though it was unclear with what mechanisms. The NaOH-OP pool was accessed using both phosphatase enzymes and oxalic acid while the residual-P pool was slightly depleted by plant uptake or transformation into a different pool through interaction with citric acid. From this study, it appears that plants are using multiple mechanisms to access multiple pools of P.

INTRODUCTION

With the growing population, it is essential that agricultural systems become resource use efficient in every aspect to ensure that food supply can meet food demand. Globally, cereal demand is expected to increase by 0.9 billion metric tons by 2050 (Bruinsma, 2003; WOAB, 2011) This needed efficiency is especially true for phosphorus (P) use efficiency as P is limiting on 30% of the world's arable crop land (Vance et al., 2003; Korkmaz et al., 2009).

Phosphorus is an essential macronutrient to plants. It is a key constituent of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in the cell nucleus (Troeh and Thompson, 1993a) and is found in adenosine triphosphate (ATP) and adenosine diphosphate (ADP) (Noggle and Fritz, 1983). If insufficient P is available to plants, their biomass or grain yield can be limited. The phosphate reserves that society relies on are expected to be depleted in the next 40-60 years (Stewart et al., 2005). Because of this, the recent increase in P-fertilizer cost, from \$221 ton^{-1} in 2002 to \$665 ton^{-1} in 2012 (USDA, 2013), is expected to continue into the future. Globally, wheat uses 16.4% of applied P-fertilizer, ranking it second only to high value fruit and vegetable crops (Heffer, 2009). Of the applied P-fertilizer, only 10-20% is recovered by plants during the growing season (Sharpley, 1986; Mclaughlin et al., 1988). Because of this, it is warranted and necessary to study strategies that will increase the ability of crops to better produce high yields with less P input.

In the soil, P is a particularly complex nutrient. Phosphorus cycling in soils is complex due to the number of forms that P can be found in. However, P can broadly be categorized into three pools; solution P, active P, and fixed P. The solution P pool is the smallest yet most readily plant available P pool; being predominantly in orthophosphate form (HPO_4^{2-} and $H_2PO_4^-$). The

active P pool releases P into the solution P pool by dissolution of soluble inorganic P and mineralization of organic P. The fixed P pool is primarily insoluble inorganic P and can contribute a small quantity of P into the active pool (Busman et al., 2002).

Within these pools, P is found in three general forms; organic P, calcium (Ca)-bound inorganic P, and iron (Fe)- or aluminum (Al)-bound inorganic P (Brady and Weil, 2008). Organic P forms include phospholipids, nucleic acids, and inositol phosphates (Tisdale and Nelson, 1975). In high pH calcareous soils, Ca is very abundant. The Ca reacts with phosphate and forms relatively insoluble calcium phosphate compounds. These calcium phosphate compounds however, are acid soluble and thus sensitive to pH changes.

Phosphorus-use efficiency is exhibited in plants through two distinct strategies (Batten, 1992; Osborne and Rengel, 2002). The first strategy, P-utilization efficiency, involves internal mechanisms that allow the plant to produce greater biomass with relatively less P taken up from the soil. These internal mechanisms include using morphological, metabolic, and physiological responses such as alternate metabolic pathways and increasing the shoot-root ratio (Hammond et al., 2004). While this strategy is useful, it has a certain limitation as the plant still has to access and uptake P in order for plant growth to occur. The second strategy, P-uptake efficiency, is defined as a plant possessing the ability to uptake relatively larger amounts of P from relatively recalcitrant soil P pools. (Föhse et al., 1991). Because P-uptake efficient plants are better able to extract a larger quantity of P from the soil they are then able to produce greater biomass or grain yield. Breeding for this strategy can help to produce cultivars that are better able to grow in the large number of soils that are typically considered low in plant available P but high in non-labile forms of P (Runge-Metzger, 1995; Manske, 2001)

Phosphorus-uptake efficiency is achieved through various chemical mechanisms in the soil. Plants release organic acids and phosphatase enzymes than react with phosphate containing compounds and subsequently make P more available to plants. Plants also modify the rhizosphere soil pH which can transform particular forms of P into more plant available forms (Hedley et al., 1982; Tarafdar, 1987; Dinkelaker et al., 1989; Hocking, 2001). The amount and type of these exudates varies between and within plant species and is also variable with environmental conditions. Plants can also form symbiotic relationships with arbuscular mychorizal fungi, essentially increasing the surface area of the roots and thus increasing access to and uptake of P (Zhu and Chen, 2002).

In calcareous soils, Dinkelaker et al. (Dinkelaker et al., 1989) and Gardner et al. (Gardner et al., 1983) found citric acid to be important in P cycling in the rhizosphere of white lupin. For maize grown in calcareous soils, Strom et al. (Strom et al., 2001) found that oxalic, malic, and citric acid to be important in the rhizosphere but did not play a significant role in the bulk soil. In a later experiment, Strom et al. (Strom et al., 2005) also characterized the effectiveness of each of these organic acids in transforming P into more available forms in calcareous soils and found that oxalic was the most effective, citric was next, and malic was least effective. These organic acids are making typically non-bioavailable P more available through three mechanisms (Gerke et al., 2000): chelation of metal-phosphate compounds, forming organic acid-phosphate-metal complexes that are soluble and thus more accessible to plants (Gardner 1983), and competing with phosphate compounds for adsorption sites on soil colloids (Hocking, 2001). Phosphatase enzymes aid in making P more plant available by removing phosphate groups from phytic acid, which is the most common form of soil organic P. In calcareous soils, alkaline phosphatase enzymes dominate (Tarafdar, 1987). To achieve internal charge balance when taking up cations

from the soil, plants excrete H^+ or HCO_3^- (Grinsted et al., 2011). Proton exudation decreases soil pH and can dissolve acid-soluble forms of inorganic P, thus making them more available to plants (Hedley et al., 1982).

Many studies have focused on P-utilization efficiency (Gardiner and Christensen, 1990; Föhse et al., 1991; Bhadoria et al., 2002; Manske et al., 2002; Wang et al., 2005). These studies have provided great insight into both the variation of P-utilization efficiency found between plants as well as varying responses to P-addition. However, much work is still called for to identify and examine the impact of root exudates on P-cycling and P-uptake by wheat.

For producers and breeders working on increasing P-uptake efficiency in high pH calcareous soils, more work is needed to establish which exudates plants are using to become P-uptake efficient as well as the exudates impact on various soil P forms. Doing so would provide valuable information to better screen and breed accessions that exhibit great abilities to extract typically non-plant available P as well as more efficiently use added P fertilizer.

METHODS

In a previous experiment, twenty-two winter wheat accessions were grown in a low P calcareous soil to determine which accessions were mostly P-uptake or P-utilization efficient. For this study, the three most P-uptake efficient and three least P-uptake efficient were used. The selected accessions were grown in rhizo-cells, a unique set-up modified from Hedley et al. (1982). The rhizo-cells have two compartments that are 5 cm in diameter and 7.62 cm tall that were connected together by adhering them with tape. The two compartments were separated by a 30 μm mesh screen. The bottom compartment was filled with the low P-calcareous soil, on which the mesh screen was placed. On top of the screen, 0.25g of wheat seed for the particular

accession was placed and subsequently covered with 1.27 cm of pure lab-grade sand (Acros Organics, 40-100 mesh). The purpose of using the rhizo-cells is that they enable the plant roots to interact with the soil, but not grow down into the soil. This both eliminates root growth and architecture as a possible P-uptake strategy and allows for the soil to be sliced and analyzed without erroneously including root tissue in the analysis.

For this study, two sets of rhizo-cells were established. The first set was replicated four times to collect pH, phosphatase enzyme, and P fractionation data. The second set was replicated three times in order to collect organic acid data. Rhizo-cells for each experiment were grown in a growth chamber for twenty-eight days after emergence. The chamber was preset to deliver 16 hours of daylight at a lighting intensity of 200 µmoles m^{-2} per s and for temperatures to be 25 ^{o}C during the day and 20 ^{o}C at night. In order to insure that plants were not nitrogen deficient and to keep the soil at near constant field capacity, each rhizo-cell was placed in a water reservoir with 1400 mg L^{-1} potassium nitrate (KNO_3) fertilizer solution.

Background physical and chemical properties were measured on the soil prior to the experiment. To do so, the soil was dried at 65° C and sieved to 2 mm. The soil was then analyzed for pH, NO_3-N, extractable P, Total P, exchangeable Aluminum, and K, SO_4-S, Ca, Mg, Fe, Zn, B, and Cu (Table 1.1). Soil pH was determined by placing a combination electrode in a 1:1 soil to water solution (Thomas, 1996). Nitrate nitrogen (NO_3-N) was determined by taking 2.5g of soil and shaking with 10 mL 1.0 *M* KCl for 15 minutes. This extract was then analyzed for NO_3-N using a Lachat Quickchem 8000 automated flow-injection analyzer (Mulvaney, 1996). Bioavailable P was determined according to the method developed by Olsen et al. (1954) in which 2.0g of soil was extracted by shaking with 40 mL of 0.5 *M* $NaHCO_3$ for 30 minutes, filtered through Whatman #2 filter paper and analyzed on an ICP-AES. Total P was measured by

completing an acid digestion of 0.5g of soil according to the EPA 3050 method and analyzing the resulting extracts with ICP-AES (EPA,1986). For exchangeable Al determination, 5g of soil was shaken with 20 mL 1.0 *M* KCl for thirty minutes, filtered, and then analyzed on an ICP-AES (Page et al., 1982). To measure SO_4-S, 10g of soil was shaken with 25 mL 0.008 *M* calcium phosphate, and then analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Spectro Ciros, Mahwah, NJ) (Schulte and Eik, 1988). Exctractable K, Mg, and Ca were determined by shaking 2.0g of soil with Mehlic 3 (M3) solution (0.015 *M* NH_4F, 0.2 *M* CH_3COOH, 0.25 *M* NH_4NO_3, 0.013 *M* HNO_3, 0.001 *M* EDTA), filtering the extract through a #2 Whatman filter paper, and then analyzing the filtered extract by ICP-AES (Mehlich, 1984). Iron, Zn, B, and Cu concentrations were determined by shaking 10g of soil with 20 mL DTPA-Sorbitol (0.005 *M* DTPA, 0.1 *M* triethanolamine (TEA), 0.01 *M* $CaCl_2$, and sorbital) for 2 hours and analyzing with ICP-AES (Lindsay and Norvell, 1978). Soil texture was determined according the hydrometer method developed by Gee and Baude (Gee and Baude, 1986).

Plant Analysis

Both plant roots and shoots were harvested and dried for 24 hours at 85°C to determine dry plant biomass yield. Replicates of plant biomass were compiled together for each experiment due to low biomass yields. To analyze for P content of the plants, the compiled samples were completely digested and the resulting solution analyzed on ICP-AES (Jones and Case, 1990). To calculate P uptake by each plant, the concentration of P (mg P kg biomass $^{-1}$) was multiplied by the combined biomass yield (kg) and divided by the number of replications in the composite sample (eq.1)

$$(1)\ P\ uptake\ (mg) = \frac{Plant\ P\ concentration \times total\ plant\ biomass}{number\ of\ replications\ in\ composite}$$

Soil Sampling

After harvesting the root sand shoots from the rhizo-cells, the soils were sliced at different depths. For the first set of rhizo-cells the upper 3.75 mm of soil in the bottom compartment (under the mesh) was sliced into five 0.75 mm increments while the second experiment was sliced into two 2.25 mm slices. To do so, rhizo-cells were placed horizontally and the soil pushed uniformly from the bottom for the designated depth. The advanced soil was then sliced with a thin wire.

Soil Analysis

To determine phosphatase content in the soil, 0.2g of soil from each depth was collected and place in a small vial without drying. The soil was then subjected to the assay method developed by Tabatabai (Tabatabai, 1994) in which 0.2 mL of toluene, 4 mL of modified universal buffer, and 1 mL p-nitrophenyl phosphate solution is added into the vial, gently mixed with the soil, and a rubber stopper placed in the vial opening. The vials were then placed in an incubator for 1 hour at 37^0 C. After incubation, the vials were removed and 1 mL of 0.5 *M* $CaCl_2$ and 4mL of 0.5 *M* NaOH were added to the soil solution. The solution was then gently swirled and filtered through a no. 2v Whatman filter paper. The filtered solution was then analyzed for color intensity using a spectrophotometer using a wavelength of 400 nm (Milton Roy Spectronic 21D). For this assay method, the amount of *p*-nitrophenol released is an indicator of the amount of phosphatase enzyme present. Standards were prepared by incubating various 0, 10, 20, 40, and 40 ug of p-nitrophenol according to the same method for the soil samples. However, the addition of 1 mL p-nitrophenyl phosphate was carried out after the solution had been incubated instead of before incubation. The measured results were then fit into a standard curve to determine the

amount of *p*-nitrophenol released. Four replicates of control soil were also prepared and the results subtracted from the experimental samples to take into account native soil phosphatase enzyme content.

Soil pH was measured by placing a combination electrode in a 1:1 soil to water solution (Thomas, 1996). Each depth was subjected to a complete soil fractionation according to Hedley et al. (Hedley et al., 1982). Slight modifications were made for this study. For each depth, 0.5 g of soil was used. The Murphy Riley method (Murphy and Riley, 1962) was used to determine inorganic P. Total P was quantified by complete digestion according to Tiesson and Moir (Tiessen and Moir, 1993) in which the digested sample was then subjected to the Murphy Riley method to determine P content. To determine organic P, inorganic P was subtracted from total P. Solution P was determined by shaking the 0.5 g of soil sample with 30 ml DI H_2O overnight with 1 resin strop (ResinTech, AMB-SS Anion Single Sheet Ion Exchange Membrane). After shaking, the resin strip was removed and rinsed with DI H_2O and then shaken with 20 mL of 0.5 *M* HCl overnight. The resin strip was then removed and the remaining solution was analyzed for inorganic P. The soil solution from the first step was then centrifuged at 10,000 rpm for 15 minutes and the supernatant decanted and discarded. The soil was then shaken with 30 mL of 0.5 *M* $NaHCO_3$ overnight, centrifuged and decanted. The supernatant was then analyzed for inorganic and organic P. This extraction represents the "loosely bound" P in the soil. To determine Fe and Al phosphate, the soil was then shaken with 30 mL 0.1 *M* NaOH overnight, centrifuged and the supernatant decanted and analyzed for both inorganic and organic P. For the next step, the soil was then shaken with 30 mL 1 *M* HCl overnight and then centrifuge and decanted. This was then analyzed for inorganic P and represents the Ca-phosphate pool. To determine residual P, a complete digestion of the soil was executed according to the EPA 3050

method (EPA, 1986). For this method, the soil was heated with concentrated HNO_3, concentrated HCl, and 30% H_2O_2. The extract was then let filtered through a no. 2 Whatman filter and the extract was analyzed for inorganic P.

For the final step, the EPA 3050 complete digestion method was used to determine residual P (EPA,1986). Also, solution P was determined by extracting with a resin strip (ResinTech, AMB-SS Anion Single Sheet Ion Exchange Membrane). For each depth, 0.5g of soil was used for the fractionation. The fractionation procedure extracts and categorized P into several fractions: solution P (with resin strip), "loosely" bound P extracted with $NaHCO_3$, Fe and Al bound P extracted with NaOH, Ca bound P extracted with HCl, and residual P which was the complete digestion of remaining soil material. The fractionation was included in this experiment to determine which pool(s) of P the P-uptake accessions are accessing.

For the second experiment 5g of soil from each of the two depths, Depth 1 (0-2.25 mm) and Depth 2 (2.25-3.75 mm), was shaken with 50 mL of 0.01 *M* NaOH for two hours. This was then centrifuged at 20,000 rpm for 20 minutes and the supernatant filtered through a 0.45 µm with the aid of a vaccum filter. This solution was then subjected to purification through 5 mL volume of Amberlite cation-exchange resin (Amberlite IR120, H^+ form, Fluka Analytical). Solution was ran through Amerblite resin with the aid of a vacuum pump and subsequently rinsed with DI H_2O. This resin purified cations from the solution. The cation free solution was then ran through 2.0 g of Dowex anion-exchange resin (Dowex 1x8 ion exchange resig, Sigma-Aldrich) and rinsed with DI H_2O. The Dowex resin was then washed with 8 mL of 1M HCl to release the organic acids into solution. The completely purified solution was then vacuum dried and reconstituted with 1mL 0.05 *M* H_2SO_4. This solution was then analyzed for malic, citric, and oxalic acid on a high-performance liquid chromatography (HPLC, Dionex 4500i; Sunnyvale, CA,

USA) with VDM-2 variable wavelength detector, GPM-gradient pump, and AS3500 autosampler. The HPLC was outfitted with an Aminex HPX-87H column (Bio-Rad, Hercules, CA), guard column (Bio-Rad, Hercules, CA), and column heater (Eppendorf CH500, Hamburg, Germany) set at 60°C. The HPLC mobile phase was 0.005 M H_2SO_4 flowing at 0.6 ml min^{-1}. Organic acids were analyzed with a wavelength of 214 nm. Peak area of the three organic acids was analyzed using PeakNet software (Version 5.21; Dionex).

Statistical Analysis

All data were analyzed using SAS software package (SAS Institute, 2009). Duncan Groupings between all accessions and within pools and depths were computed using Means statements with the GLM procedure with an alpha value of 0.05 being significant. Single Pearson Correlation coefficients were computed using the CORR procedure for single correlations between P-uptake and the phosphorus pools, exudates, and biomass for two depths (Table 1.4) and between exudates and P-pools (Table 1.5).

RESULTS/DISCUSSION

Plant growth and phosphorus-uptake

From a previous study, accessions 2, 4, and 6 were characterized as mostly P-uptake efficient while accessions 5, 9, and 21 were identified as P-uptake inefficient. However, no accession was characterized as showing solely P-uptake efficiency. Instead, accessions were characterized as P-uptake efficient but still showing signs of P-utilization efficiency.

Results from this study are congruent with that as accessions 5, 4, and 9 had both the highest yield and P-uptake (Table 1.2) with accession 4 being the only P-uptake efficient

accession from the previous study to exhibit this trait for the current study. Biomass ranged from 114.7 mg to 573.6 mg and P-uptake from 0.2 mg to 0.47 mg. P-uptake was significantly correlated with biomass (R^2=0.95). The focus of this discussion is to ascertain how P was accessed among the various wheat accessions used in the experiment.

Chemical Mechanisms in the Soil

Because there is normally much more nutrient cycling and microbial activity in the soil directly adjacent with roots, the top three depths (0-2.25 mm) were compiled for analysis and were deemed the "rhizosphere" and the bottom two depths (2.25 mm-3.75 mm) were compiled and deemed the bulk soil. This is consistent with the concept that the rhizosphere soil is the approximately 2.0 mm of soil surrounding plant roots (Pinton et al., 2001).

Rhizosphere Soil (Depth 1)

For the rhizosphere (0-2.25 mm), P-uptake was significantly negatively correlated with $NaHCO_3$-IP (Table 1.3). This indicates that plants are indeed accessing this pool. This reaffirms the common practice of utilizing $NaHCO_3$ as a suitable extractant when trying to measure plant available P in semi-arid and arid soils. The NaOH-OP fraction appears to be influenced by oxalic acid and phosphatase enzymes (Table 1.4). Recall that this fraction represents primarily organic P assocated with Fe and Al, likely Al and Fe-phytate. Therefore, oxalic acid could be chelating Fe and Al located on the phytic acid ring, which makes phytate more bioavailable to microorganisms that excrete phytase enzyme. Increasing the hydrolysis of phytate ultimately results in the release of P into solution where plants can better access it (Hocking, 2001; Tarafdar and Claassen, 1988). The ability of oxalic acid to chelate metals is indicated by the complex formation constant (log (K_f)) for oxalic acid with each metal ion; Ca=3.0, Fe(II)= >4.7, Fe(III)=

9.4, and Al(III)= 7.26 (Furia, 1972). For the rhizosphere, citric acid was also seen negatively correlated with residual-P (Table 1.4). This suggests that this organic acid could potentially be making this residual pool more available for plants. Parker et. al (2005) stated that citric acid has a great ability to alter metal speciation, which ultimately affects P solubility in soils. This is seen by its complex formation constant with the following metals; Al (III): pKa=11.7, Ca: pKa= 3.5, Fe (II): pKa=3.2, Fe(III): pKa=11.85 (Perrin et al., 1981).

Bulk Soil (Depth 2)

The rhizosphere is typically believed to possess the most nutrient cycling and activity. For this study, this appears to be true as no variables were significantly negatively correlated with P-uptake. However, $NaHCO_3$-OP was significantly positively correlated with P-uptake (Table 1.4). This suggests that this pool is not bioavailable for plants to use. While it does not appear that P is being made more available overall, citric acid is negatively correlated with residual-P (Table 1.4) as was also seen in the rhizosphere. This indicates that while citric acid is not affecting P-uptake immediately, it could potentially have an impact by making the residual-P more bioavailable over longer periods of time.

While pH was not significantly correlated with P-uptake or any of the P-pools, each accession did decrease their pH relative to the soil. Accession 9, one having high P-uptake, had one of the greatest drops in pH (Figure 1.1, Table 1.5). This dramatic drop in pH could potentially be making the Ca-phosphate pool (HCl-P) more soluble and thus could lead to greater P-uptake. Although not significant, HCl-P and pH was lower in the rhizosphere than the bulk soil for nearly every accession (Table 1.5), suggesting that a decrease of pH in the rhizosphere is making this pool more plant available.

CONCLUSION

From this study, it can be concluded that plants are accessing the $NaHCO_3$-IP pool though it is unclear through what mechanisms. It can also be determined that plant exudation of oxalic acid, citric acid, and phosphatase enzymes are contributing to enhanced nutrient cycling and possibly making P more bioavailable. Additional work is warranted to study the long-term impact of the exudates on P-pools and the subsequent impact to plant P-uptake.

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TABLES AND FIGURES

Table 4.1 Chemical characteristics for Richfield clay loam Series soils.

Soil Series	pH	NO_3-N	Extractable P	Total P	Exch. Al	K	SO_4-S	Ca	Mg	Fe	Zn	B	Cu
			----------mg kg^{-1}----------										
Richfield	7.9	1	8[§]	2131[¥]	0.1	564	7	4032	502	12.3	0.3	0.7	1.2

§ Bicarbonate extractable P
¥ EPA 3050 Total P

Table 4.2 Plant mean biomass and phosphorus (P) uptake for each accession.

Accession	Mean Biomass (mg)	P-uptake (mg)
(5) Jerry¥	573.6a*	0.47
(4) MO4*5109¥	485.3a	0.41
(9) OK91G103 (Chisholm-T)±	449ab	0.47
(21) OCW00S063S-1B¥	413.6ab	0.41
(6) SD06069¥	341ab	0.36
(2) P03207A-7¥	114.7b	0.20

*Indicates significant difference between accession at p=0.05
¥ Field tolerant to acidity, no 4DL marker
± Tolerant to acidity, 4DL marker present

Table 4.3 Correlation Coefficients between phosphorus (P) uptake and soil P pools, exudates, and biomass determined from rhizo-cells after 28 days of wheat growth. Phosphorus pools, pH, and exudates correlated were the average of 0-2.25 mm (depth 1) and 2.25-3.75 mm (depth 2).

	Inorganic Phosphorus				Organic Phosphorus								
Depth	Resin	0.5 *M* $NaHCO_3$	0.1 *M* NaOH	1.0 *M* HCl	0.5 *M* $NaHCO_3$	0.1 *M* NaOH	EPA 3050 Digestion	pH	Phosphatase Enzyme	Oxalic Acid	Citric Acid	Malic Acid	Biomass
1	-0.09	-0.84*	-.54	0.12	0.41	-0.26	0.47	-0.33	0.09	-0.26	-0.12	-0.34	0.95*
2	-0.47	-0.62	-0.28	0.12	0.76*	0.04	0.58	-0.46	-0.16	-0.18	-0.57	-0.40	

* Indicates significances at p=0.10

Table 4.4 Correlation Coefficients between exudates and soil phosphorus (P) pools determined from rhizo-cells after 28 days of wheat growth. Phosphorus pools, pH, and exudates correlated were the average of 0-2.25 mm (depth 1) and 2.25-3.75 mm (depth 2).

		Inorganic Phosphorus				Organic Phosphorus		
		Resin	0.5 *M* $NaHCO_3$	0.1 *M* NaOH	1.0 *M* HCl	0.5 *M* $NaHCO_3$	0.1 *M* NaOH	EPA 3050 Digestion
Accession	Depth	----------mg P kg^{-1} soil----------						
pH	1	-0.59	--0.10	-0.32	0.50	-0.12	0.31	0.41
	2	-0.48	0.76	-0.31	0.48	-0.17	-0.46	0.31
Phosphatase Enzyme	1	0.03	-0.12	0.07	0.06	0.63	-0.87*	-0.45
	2	0.68	0.31	0.35	-0.55	-0.59	0.43	-0.43
Oxalic Acid	1	-0.57	-0.05	0.15	0.21	0.19	-0.74*	-0.06
	2	-0.27	-0.65	-0.28	0.43	-0.02	-0.15	-0.11
Malic Acid	1	0.08	0.44	0.28	-0.06	-0.50	-0.19	0.06
	2	0.21	-0.36	0.18	-0.03	-0.22	0.21	-0.55
Citric Acid	1	0.91*	0.55	0.50	-0.56	0.02	0.02	-0.77
	2	-0.41	0.79	0.62	-0.05	-0.25	0.12	-0.75*

* indicates significance at p=0.10

Table 4.5 Soil phosphorus forms and pH after 28 days of wheat growth in rhizo-cells. Values are average of 0-2.25 mm (depth 1) and 2.25-3.75 mm (depth 2) for each accession.

		Inorganic Phosphorus				Organic Phosphorus			
		Resin	0.5 *M* $NaHCO_3$	0.1 *M* NaOH	1.0 *M* HCl	0.5 *M* $NaHCO_3$	0.1 *M* NaOH	EPA 3050 Digestion	pH
Accession	Depth	----------mg P kg^{-1} soil----------							
(5) Jerry	1	1.31ab	1.73a	5.79a	178.14a	0.01a	460.24a	1545.37a	6.99a
	2	1.21A	1.63A	5.65A	184.86A	0.28A	438.16A	1548.19A	7.08A
(4) MO4*5109	1	0.94b	1.34a	4.78a	205.17a	0.24a	412.94a	1395.35a	7.01a
	2	1.50A	1.09A	4.57A	208.44A	0.20A	384.66A	1365.10A	7.02A
(9) OK91G103	1	4.07a	2.28a	5.52a	179.69a	0.17a	447.58a	1234.69a	6.88a
	2	2.15A	2.37A	5.61A	181.68A	0.25A	414.81A	1248.30A	6.9A
(21) OCW00S063S-1B	1	2.44ab	2.51a	6.24a	180.04a	0.08a	415.32a	1335.94a	6.84a
	2	1.74A	2.40A	5.24A	182.16A	0.01A	427.77A	1498.87A	6.97A
(6) SD06069	1	2.47ab	2.81a	7.27a	162.07a	0.12a	439.0a	1120.67a	6.90a

	2	2.22A	1.95A	6.70A	161.99A	0.11A	482.04A	1084.34A	6.88A
(2) P03207A-7	1	2.76ab	3.91a	6.67a	180.23a	0a	460.63a	1233.04a	7.0a
	2	2.10A	3.09A	5.82A	184.63A	0A	410.14A	1159.40A	7.13A

Data with the same lower case letter indicates no significant difference between the same P-fraction of other accessions for depth 1 (p=0.05). Data with same upper case letter indicates no significant difference between the same P-fraction of other accessions for depth 2 (p=0.05).

Table 4.6 Soil concentrations of phosphatase enzyme, oxalic acid, citric acid, and malic acid extracted from rhizo-cells after 28 days of wheat growth. Values shown are the average of 0-2.25 mm (depth 1) and 2.25-3.75 mm (depth 2).

Accession	Depth	Phosphatase Enzyme (μmol *p*-nitrophenol g^{-1} soil/ min)	Oxalic Acid (μmol g^{-1} soil)	Citric Acid (μmol g^{-1} soil)	Malic Acid (μmol g^{-1} soil)
(5) Jerry	1	0.049627a	0.026746a	0a	0.005267a
	2	0.048149a	0.035713ab	0a	0.008558a
(4) MO4*5109	1	0.073253a	0.036709a	0a	0a
	2	0.059692a	0.068815a	0.025092a	0.073181a
(9) OK91G103	1	0.06408a	0.0214a	0.003328a	0a
	2	0.066267a	0.019482b	0.003879a	0a
(21) OCW00S063S-1B	1	0.077963a	0.036945a	0.002726a	0.031992a
	2	0.077459a	0.031991ab	0a	0.014682a
(6) SD06069	1	0.075328a	0.034673a	0.002559a	0a
	2	0.078188a	0.0503ab	0.030363a	0.008558a
(2) P03207A-7	1	0.056147a	0.030071a	0.001921a	0.018577a
	2	0.061939a	0.038275ab	0.021418a	0.040145a

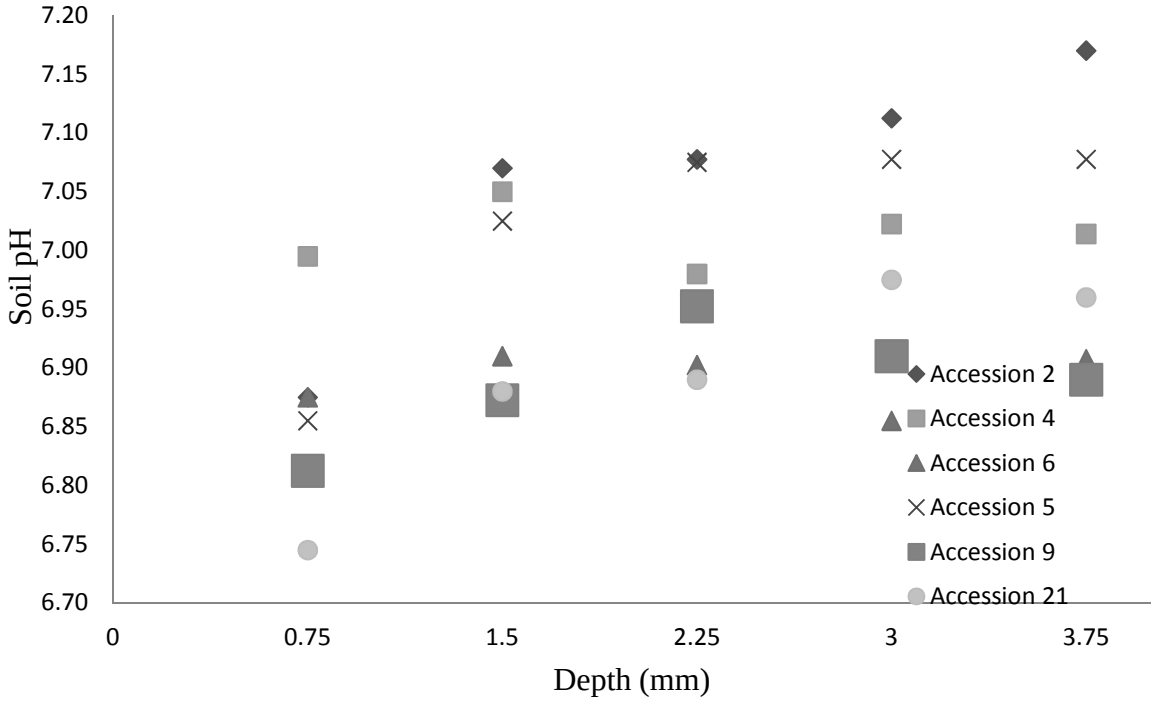

Figure 4.1 Change in soil pH by depths for each accession.

VITA

PATRICK RYAN BELL

Candidate for the Degree of

Master of Science

Thesis: INCREASING PHOSPHORUS EFFICIENCY: AN INVESTIGATION OF PHOSPHORUS UPTAKE MECHANISMS IN THE RHIZOSPHERE OF WHEAT

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Title of Study: INCREASING PHOSPHORUS EFFICIENCY: AN INVESTIGATION OF PHOSPHORUS UPTAKE MECHANISMS IN THE RHIZOSPHERE OF WHEAT

Pages in Study: 111 Candidate for the Degree of Master of Science

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Scope and Method of Study: Phosphate reserves are declining in both quality and quantity and are expected to be depleted in the next 40-60 years. Increasing phosphorus (P)-use efficiency in agricultural systems is therefore warranted. The objectives of this study were to: screen twenty-two winter wheat accessions for phosphorus efficiency in acid and calcareous low P soils and characterize the them as P-uptake or P-utilization efficient or inefficient, and the second objective was to analyze the rhizosphere of the P-uptake efficient accessions to determine what mechanisms these plants utilize to access typically non-plant available P. To screen the accessions, plants were grown in low, medium, and high P acid and calcareous soils for 28 days. Biomass and P-uptake were measured and analyzed to characterize the accessions. Three P-uptake efficient and three P-uptake inefficient accessions from each soil type were then grown for 28 days in rhizo-cells. Five soil depths from 0-3.75mm at increasing distance away from the roots were analyzed for organic acids, phosphatase enzymes, soil pH, and P forms.

Findings and Conclusions: In the acid soil, six accessions were characterized as primarily P-uptake efficient and three accessions as P-utilization efficient. In the calcareous soils, six accessions were characterized as primarily P-uptake efficient while four were characterized as predominantly P-utilization efficient. For the accessions chosen for the rhizosphere analyses in the acid soil, oxalic and citric acid as well as pH changes were identified as the mechanisms being utilized by the plants to access relatively non-labile P. The pools impacted by these mechanisms were the NaOH-IP, NaOH-OP, and HCl-P pools. For the calcareous soil rhizosphere analyses, oxalic and citric acid were again observed as important exudates as well as alkaline phosphatase enzymes. The pools impacted by these exudates were the $NaHCO_3$-IP, the NaOH-OP, and the residual-P fractions. From this study it is concluded that plants exhibit varying responses and abilities to handle low-P environments. Plants use multiple exudates to access multiple fractions of P that are typically recalcitrant. Additional work is needed to identify genes controlling the release of the identified exudates in order to effectively incorporate these P-uptake efficiency mechanisms into new accessions.

ADVISER'S APPROVAL: Dr. Chad Penn