# Physiological and genetic control of phytic acid in diverse sets of wheat (*Triticum aestivum* L.)

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## ABSTRACT

Phytic acid (PA) is the main source of phosphorus storage in plants. Since the molecule has a negative charge, it creates complexes with important minerals such as Cu, Zn, Co, Mn, Mg, Fe and Ca. It is therefore regarded as an antinutrient because monogastric animals do not have the necessary enzymes to break the bonds. This leads to major issues such as micronutrient deficiencies in the population, all the unused phosphorus is excreted and eventually reaches water bodies causing eutrophication. Up to 60-85 % of P from soil is stored as PA in grains, hence all this P is removed from the soil at harvest and this removed P has a cost, which has been estimated in billions. Hence, even small reductions in the PA concentrations could represent more efficient and nutritious crops as well as important money savings.

In this study we analysed wheat samples grown in a hydroponic system and evaluated the effect of P and Zn treatments on its concentration in leaf and grain (Chapter 2). Treatments had significant effect over PA and other mineral concentrations in grain and leaf samples. We observed significant relationships between leaf and grain PA and mineral concentrations indicating that some predictions could be made from a single and simple analysis in leaves. In Chapters 3 and 4 we describe a huge variability in PA concentrations in diverse genotypes and environments. We found significant differences between the genotypes, environments and their interactions. Moreover, phytate to mineral molar ratios were calculated and the potential impact over the bioavailability of Ca, Fe and Zn is discussed. The results obtained here highlight the importance of PA determination as an important trait to be looked at when

breeding or searching for mineral enhanced varieties. As we observed, some genotypes with high concentrations of Fe and Zn had also high concentrations of PA. High PA ratios will inevitably affect the bioavailability of Fe and Zn.

A great amount of work has been done over the past years, but there are still gaps of knowledge to be filled, such as the transport and loading of P into seeds, the genetic control of P translocation from vegetative tissues to seed, the heritability of P and PA traits, among others. This work aims to set the basis for further and more specialized studies looking into developing new low phytate varieties.

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To my beloved parents and sisters who never stop believing in me, although far away always encouraged, loved and motivated me to keep going. For all they have sacrificed for me to come this far. Todos mis logros son para y gracias a ustedes, los amo.

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# ABBREVIATIONS

| | |
|---|---|
| °C | degree Celsius |
| ANOVA | Analysis of Variance |
| AOAC | Association of Official Analytical Chemists |
| ATP | Adenosine triphosphate |
| BBCH | Biologische Bundesanstalt, Bundessortenamt and CHemical industry |
| Ca:PA | Calcium to Phytic Acid ratio |
| Ca:Zn | Calcium to Zinc ratio |
| CEC | Cation Exchange Capacity |
| cm | centimetre |
| CRM | Certified Reference Material |
| DAP | Days After pollination |
| delta-Abs | delta absorbance (difference in absorbance) |
| DNA | Deoxyribonucleic Acid |
| dS m-1 | deciSiemens per metre |
| DW | Dry Weight |
| DWB | Dry Weight Basis |
| EC | Electrical Conductivity |
| FAO | Food and Agriculture Organization |
| FAOSTAT | Food and Agriculture Organization Corporate Statistical Database |
| FNB/IOM | US Food and Nutrition Board/Institute of Medicine |
| FW | Fresh Weight |
| g | gram |
| g $mol^{-1}$ | gram per mole |
| GHI | Global Hunger Index |
| GLPA | Goldhull Low Phytic Acid |
| GRAS | Generally Recognized As Safe |
| GS | Growth Stage |
| GYD | Grain Yield |
| h | hour |
| HAA | Heterocyclic Aromatic Amines |
| HCl | Hydrochloric acid |
| HF | Hydrofluoric acid |
| HL | Wolfgang & Lantzsch Method |
| HPLC | High Performance Liquid Chromatography |
| HvST | *Hordeum vulgare* L. sulphate transporter |
| ICP-MS | Inductively Coupled Plasma Mass Spectrometry |
| IP | Inositol Phosphate |
| IP3 | *myo*-Inositol trisphosphate |
| IP4 | *myo*-Inositol tetrakisphosphate |
| IP5 | *myo*-Inositol pentakisphosphate |
| IUPAC | International Union of Pure and Applied Chemistry |
| kg | kilogram |

| | |
|---|---|
| kg ha-1 | kilogram per hectare |
| KOH | Potassium hydroxide |
| L | Litre |
| LDPE | Low Density Polyethylene |
| LOD | Limit of Detection |
| LOQ | Limit of Quantification |
| LPA | Low Phytic Acid |
| LRM | Laboratory Reference Material |
| LSD | Least Significant Difference |
| mg | milligram |
| mg $kg^{-1}$ | milligram per kilogram |
| min | minute |
| mL | millilitre |
| mm | millimetre |
| mmol L-1 | millimole per litre |
| MNM | Micronutrient Malnutrition |
| mol | mole |
| MPa | megaPascal |
| mRNA | messenger RNA |
| MW | Molecular Weight |
| MΩ | megohm |
| N | Normality |
| n | number of values/samples |
| N | nitrogen |
| n.d | no date |
| NA | Not Applicable |
| ND | Not Determined |
| NEPZ | North-Eastern Plains Zone |
| NFT | Nutrient Film Technique |
| NIST | National Institute of Standards and Technology |
| NLM | U.S. National Library of Medicine |
| nm | nanometre |
| NMV | Number of Missing Values |
| NOBS | Number of Observations |
| NWPZ | North-Western Plains Zone |
| OC | Organic Carbon |
| PA | Phytic Acid |
| PA:Ca | Phytic Acid to Calcium ratio |
| PA:Cu | Phytic Acid to Copper ratio |
| PA:Fe | Phytic Acid to Iron ratio |
| PA:Zn | Phytic Acid to Zinc ratio |
| PAGE | Polyacrylamide gel electrophoresis |
| PAxCa:Zn | Phytic Acid multiplied by the Calcium to Zinc ratio |
| PE | Polyethylene |
| PEEK | Polyetheretherketone |
| PES | Polyethersulfone |

| | |
|---|---|
| PFA | Perfluoroalkoxy |
| Phytic Acid-P | Phytic Acid-Phosphorus |
| Phytic Acid-P | Phytic Acid Phosphorus |
| Pi | Inorganic Phosphorus |
| PTFE-TFM | Modified PTFE (Polytetrafluoroethylene) |
| QTL | Quantitative Trait Loci |
| RNA | Ribonucleic Acid |
| RO | Reverse Osmosis |
| rpm | revolutions per minute |
| RRes | Rothamsted Research |
| RSD | Relative Standard Deviation |
| SD | Standard Deviation |
| SE | Standard Error |
| SOP | Standard Operating Procedure |
| SRM | Standard Reference Material |
| STD | Standard |
| t ha-1 | tonne per hectare |
| TAG | Trace Analysis Grade |
| TGW | Target Grain Weight |
| UoN | University of Nottingham |
| UV-Vis | Ultraviolet-Visible |
| v/w | volume/weight |
| Var | Variance |
| VL | Vaintraub & Lapteva method |
| W | watt |
| WF | White Flour |
| WG | Wholegrain |
| WHO | World Health Organization |
| WT | Wild Type |
| µg | microgram |
| µmol L-1 | micromole per litre |

# 1 INTRODUCTION

## 1.1 PHYTIC ACID CHEMISTRY AND MOLECULAR STRUCTURE

The first information on phytate dates from 1855 to 1856 when Hartig reported small particles similar to potato starch grains present in plant seeds, it was later discovered that these particles were free from starch and were actually rich in phosphorus, calcium and magnesium and was assumed they served as reserve nutrient for the germination of seeds (Hartig, 1855, 1856; Pfeffer, 1872).

Later it was proposed the molecular structure for phytate as *myo*-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate (Anderson, 1914), which was then confirmed by several methods (Barrientos & Murthy, 1996; Emsley & Niazi, 1981; Johnson & Tate, 1969) and is still valid to the present date. When isolating phytic acid from wheat grain, it was noted that the molecule was, aside from sodium, associated with iron, potassium, calcium and magnesium ions (Johnson & Tate, 1969).

Inositol phosphates are formed by an inositol ring and at least one phosphate group, *Myo*-inositol is the most widely form present in nature and is also the most relevant form in terms of nutrition (L. Bohn et al., 2008). *Myo*-inositol is one of the nine possible stereoisomers of cyclohexanehexol for which “Ins” is a common abbreviation approved by the IUPAC (Nomenclature Committee of the International Union of Biochemistry, 1989). Then, *myo*-Inositol hexakisphosphate is the name for the compound in which all six hydroxyl groups of *myo*-inositol are

esterified as phosphates, this compound is usually known as phytic acid and is the name commonly used for the free acid form of *myo*-inositol hexakisphosphate. Table 1-1 describes full names and abbreviations of *myo*-inositol phosphates.

Table 1-1 The *myo*-inositol phosphates and their accepted abbreviations.

| **Full name** | **Number of phosphate groups** | **IUPAC abbreviation** | **Common abbreviation** |
|---|---|---|---|
| *myo*-Inositol | 0 | Ins | Ins |
| *myo*-Inositol monophosphate | 1 | Ins*P*$_1$[b] | IP$_1$ |
| *myo*-Inositol bisphosphate | 2 | Ins*P*$_2$ | IP$_2$ |
| *myo*-Inositol trisphosphate | 3 | Ins*P*$_3$ | IP$_3$ |
| *myo*-Inositol tetrakisphosphate | 4 | Ins*P*$_4$ | IP$_4$ |
| *myo*-Inositol pentakisphosphate | 5 | Ins*P*$_5$ | IP$_5$ |
| *myo*-Inositol hexakisphosphate | 6 | Ins*P*$_6$ | IP$_6$ |
| Diphospho-*myo*-inositol tetrakisphosphate | 6 | PP-Ins*P*$_4$ | PP-IP$_4$ |
| Diphospho-*myo*-inositol pentakisphosphate | 7 | PP-Ins*P*$_5$ | IP$_7$ |
| Bis-diphospho-*myo*-inositol tetrakisphosphate | 8 | [*PP*]$_2$-Ins*P*$_4$ | IP$_8$ |

[a] The italicization of the P denotes its use as an abbreviation for phosphate, rather than the chemical symbol for phosphorus.

[b] although it is not explicitly stated, we infer that InsP (without a numeric subscript) is actually the IUPAC-preferred abbreviation for *myo*-inositol monophosphate. However, we recommend Ins*P*$_1$, to avoid confusion with 'InsP', which is sometimes incorrectly used as a collective abbreviation for inositol phosphates.

From: (Shears & Turner, 2007)

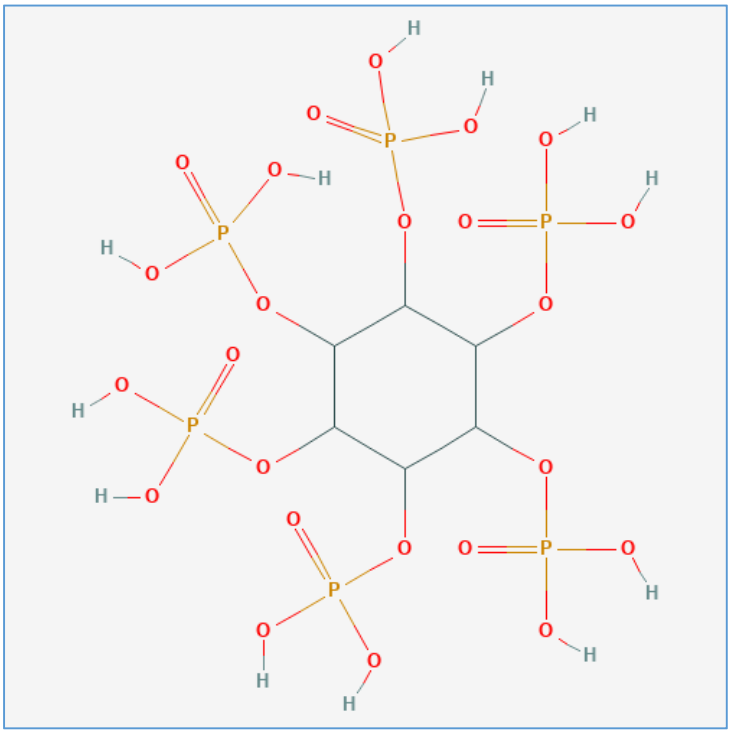


Figure 1-1 Chemical structure of phytic acid. From National Center for Biotechnology Information (2020).

The word phytate is used for any salt of phytic acid. Phytic acid (PA) can form salts with polyvalent cations (e.g. Iron) making it an insoluble salt and with monovalent cations to form a soluble salt (e.g. Sodium phytate). Nevertheless, phytate will precipitate out of solution, in a pH-dependent manner. In most cases, *myo*-inositol hexakisphosphate exists as a salt, in both precipitated and dissolved forms, and can thus be termed phytate (Shears & Turner, 2007).

Phytic acid has a molecular weight of 660.04 g $mol^{-1}$ and the chemical formula $C_6H_{18}O_{24}P_6$. Figure 1-1 shows the schematic representation of phytic acid structure.

## 1.2 ANALYTICAL METHODS FOR DETECTION AND QUANTIFICATION OF PHYTATES

Several methods have been used to quantify phytic acid (PA), the majority are derived from the ferric chloride titration method (Heubner & Stadler, 1914). Their method involves extracting the pulverized plant material with 2 % hydrochloric acid for three hours, filtered and then precipitated with ferric chloride in acid solution, resulting in the formation of insoluble ferric phytate. Ammonium thiocyanate is used as indicator of the ferric thiocyanate. This last one forms after all the ferric phytate has been precipitated. However, this last one forms a colloidal precipitate, which clouds the endpoint making it not sharp.

To overcome this difficulty, Harris & Mosher (1934) titrated the solution beyond the end point, filtered and matched the colour with that of a blank. This way the colloidal ferric phytate is removed and the measurements are more accurate. It had the disadvantage of not being adequate for coloured extracts.

Other authors, instead tried heating up the phytic acid solution with known quantities of $FeCl_3$ and afterwards separated the ferric phytate precipitate, then the excess of iron was measured colourimetrically as thiocyanate. The method allowed dealing with smaller samples and it was suitable for materials yielding coloured extracts (Young, 1936). These methods are called indirect methods because they are based on the stoichiometric relationship between phytate and other cations that are easier to measure. Direct measurements are based on the determination of phosphorus or inositol in phytic acid or its quantitative hydrolytic

products, for example, spectrophotometry, NMR spectroscopy and inductively coupled plasma mass spectrometry (ICP-MS).

McCance & Widdowson (1935) determined the phytate by estimation of the amount of phosphorus present in the ferric phytate precipitate. Phytate is extracted using HCl, precipitated as ferric phytate, and then the P in the precipitate is the estimated after incineration with sulphuric-perchloric acid. Common (1940) followed a similar approach, they determined the phytic acid content in poultry feeding stuff by extracting it with HCl and afterwards quantified the phosphorus colourimetrically using the method proposed by Fiske & Subbarrow (1925). Implementing these modifications allowed them to process a larger number of samples simultaneously.

Makower (1970) in her studies on pinto beans concluded that the indirect methods were not suitable for determination small quantities of phytic acid, such as those in immature beans. The evidence suggested the results from the ferric hydroxide ($Fe(OH)_3$) procedure were similar but faster than the wet-ashing methods and it also allowed the removal of interfering material. A year later, Wheeler & Ferrel (1971), modified McCance-Widdowson's method using trichloroacetic acid to extract PA from wheat. Phytic acid was calculated from the iron precipitated using a 4 Fe:6 P molecular ratio and suggested the possible use of the method for other wholegrains barley, rice, oats, milo and corn.

Then Harland & Oberleas (1977) presented their method which involved the use of an ion exchange resin. They analysed vegetable protein products and confirmed the ion-exchange method gave lower concentrations and less variation in the values between replicates than the iron precipitation method. Later in 1988, the AOAC (method 986.11) adopted their approach as the official method for determination of PA which is still used to this date.

Some doubts emerged regarding the specificity of the Harland and Oberleas method. Given that the precipitate is not only PA (de Boland et al., 1975) but rather a mixture of inositol phosphates, although it was proposed that inositols – mono, -di and –tri phosphates were not precipitated due to their high solubility and hence not quantified (Møllgaard, 1946).

Consequently other methods were proposed, like the one by O'Neill (1980), which used phosphorus-31 Fourier transform nuclear magnetic resonance spectrometry. The method was specific for inositol hexaphosphate and was able to discriminate lower inositol phosphates as well as inorganic phosphates.

Uppström & Svensson (1980) used a phytase from wheat to digest PA after its extraction. The solution was then measured spectrophotometrically at 780 nm and PA was calculated using a correction factor of 3.55.

In the upcoming years, more methods emerged based on combinations or modifications from the previous ones. For instance, Graf & Dintzis (1982) combined and modified Harland and Oberleas, 1977 procedure, with the HPLC

method proposed by Tangendjaja (1980) which used a μBondapack C18 column, and may be the first record of the use of HPLC for PA determinations.

Sandberg & Ahderinne (1986) successfully quantified and separated inositol tri-, tetra-, penta- and hexaphosphates in foods and intestinal contents using HPLC. The authors concluded that the values of phytate analysed by HPLC method differed from those obtained by the iron precipitation method. In the same year Harland & Oberleas (1986), used an anion-exchange column to wash the acid extract. Presuming the impurities are washed away, the eluate is then digested to inorganic phosphate (Pi) and on this basis; the PA content is calculated to be 28.2 % x inorganic phosphate value.

However, this assumption would be valid only for unprocessed grains and legumes since the processed foodstuffs contain considerable amounts of lower phosphorylated inositols such as IP5, IP4, IP3 and possibly di- and monophosphates and these are incorrectly included in the PA determinations (Lehrfeld, 1989; Phillippy et al., 1988)

In 1988 the AOAC (Association of Official Analytical Chemists) adopted as its official method the one described and modified by Harland & Oberleas (1977, 1986) for determination of phytic acid (AOAC International, 1990), it was later acknowledged that this method gave erroneous amounts of phytic acid leading to an overestimation. To investigate this, Lehrfeld & Morris (1992) compared the two methods, the AOAC official and the HPLC method in a human diet. Their findings

indicated that the AOAC method overestimated the phytic acid content by factor of 3 to 4.2 or even up to a 20 % attributed to the high proportions of lower phosphorylated inositols. Concerning the samples containing over 83 % of IP6, they found that both methods were in reasonable agreement.

Fast-forwarding to the last 20 years, more and more analytical techniques were developed. Examples are colorimetric determinations, synchronous fluorescence, isotachophoresis, high-performance ionic chromatography, and high-performance liquid chromatography.

The anion-exchange liquid chromatography with conductivity detection had the advantage of not requiring a prepurification step making it a faster procedure (Talamond et al., 2000). The new techniques allowed researchers to separate and differentiate isomeric forms of inositol phosphates, Chen & Li (2003) reported 27 peaks in the in-house reference standard solution for all 35 possible InsP2–InsP6 isomers (excluding enantiomers). Dost & Tokul (2006) used an HPLC and UV-Vis detection system to monitor the decrease of the iron(III)–thiocyanate coloured complex. The synchronous fluorescence method developed by Chen (2009) showed good agreement with the UV-spectrophotometry method, but the selectivity had to be improved according to the authors.

Nowadays, researchers are looking after high-throughput methods that could process a large number of samples whilst being accessible, reliable, fast, accurate and reproducible at the minimum cost. Examples of these are described in Raboy

(2017) who analysed modifications to two methods; three variants of the "VL method" (Vaintraub & Lapteva, 1988) and two variants of the "HL method" (Haug & Lantzsch, 1983). No method on its own was consistently acceptable although some of the variants of the methods approached the accepted measures of reproducibility (Horwitz ratio of 2.0). The "VL method" variants (extraction with 0.8 N HCl, 0.8 N HCl + 10% $Na_2SO_4$ and 0.8 N HCl + NaCl) overestimated the amount of PA but it was suggested that this difficulties could be overcome by routine plant breeding tests. The "HL method" variant where the samples were extracted adding 10 % $Na_2SO_4$, met the criteria for an accurate and reproducible, high-throughput, low cost and low-tech method.

In a further study, Raboy (2020) proposed that the "HL method" could be used along PAGE analysis. The authors stated that the "HL method" yielded total inositol phosphates values close to those obtained by HPLC whereas a PAGE analysis could provide a good estimate of the lower inositol phosphates. Given its low cost and accessibility, this system has the potential to be considered as an alternative to HPLC methods.

In the investigation presented here the analysis of wheat grain and leaf tissue was conducted using a simple and high-throughput method developed by McKie & McCleary (2016). One of the advantages of this method is that it is specific for measurement of phosphorus released as "available phosphorus" from phytic acid, *myo*-inositol $(phosphate)_n$ and monophosphate esters by phytase and alkaline phosphatase.

The method involves the acid extraction of phytic acid followed by dephosphorylation with a phytase specific for phytic acid (Ins$P_6$) and the lower phosphorylated forms (Ins$P_2$, Ins$P_3$, Ins$P_4$, Ins$P_5$). Afterwards, an alkaline phosphatase is used to release the final phosphate group from Ins$P_1$. The phosphate released is measured employing a modified molybdenum blue assay and calculated as total phosphorus in the original sample, and as phytic acid using the factor 0.282.

This method assumes that all the phosphorus released comes from phytic acid therefore is most suitable for non-processed samples for which phytic acid comprises at least 97 % of total inositol phosphates. Overestimations of phytic acid may happen if used in processed foods and feeds, which can contain higher levels of some lower *myo*-inositol phosphate forms (i.e. Ins$P_{3\text{-}5}$).

## 1.3 IMPORTANCE OF PHYTIC ACID

*Myo*-inositol and *myo*-inositol phosphates have a wide variety of physiological functions in both animal and plant cells such as (reviewed in Loewus (2001)):

- ion uptake; *myo*-inositol-dependant sodium uptake (Nelson et al., 1999),
- cell wall biogenesis; *myo*-inositol oxidation pathway (F. A. Loewus, 2006; F. A. Loewus & Loewus, 1983; M. W. Loewus & Loewus, 1974)),
- methylation and isomerization (Bohnert & Jensen, 1996; Popp et al., 1997),

- conjugation (Cohen & Slovin, 2001),
- galactosyloligosaccharide and galactosyl cyclitol biosynthesis (Obendorf, 1997; Peterbauer et al., 1998; Peterbauer & Richter, 1998),
- phosphoinositide biosynthesis and phospholipid signalling (Coté & Crain, 1993; Munnik et al., 1998; Stevenson et al., 2000),
- glycosylphosphatidylinositol and membrane anchoring (Nakazato et al., 1998; Perotto et al., 1995),
- formation of *myo*-inositol phosphates including phytic acid (Cosgrove, 1980; Graf, 1986),
- pyrophosphorylated *myo*-inositol polyphosphates (Shears, 1998).

Phytate is ubiquitous in eukaryotic cells and it regulates many physiological functions like stress response, development, phosphate sensing and homeostasis, DNA repair, RNA editing and mRNA export Raboy (2007) among many others Shears (2001).

There is some evidence indicating that phytate and its hydrolysates might have an anticarcinogen effect in animal cells, however its mechanisms are not yet fully understood (X. Liu et al., 2020; Prasad Pallem et al., 2020; Saad et al., 2013). Several reviews have discussed the benefits of phytate as a chemopreventive and its potential use as treatment against cancer and a wide set of other diseases

(Anekonda et al., 2011; Bačić et al., 2010; Brehm & Windhorst, 2019; Omoruyi et al., 2020; Silva & Bracarense, 2016; Vucenik, 2019).

There are even some clinical trials recently completed or ongoing assessing its use as a novel treatment for bipolar disorder (ID: NCT02081287) and in the prevention of progression of the cardiovascular calcifications (CALCIFICA) results are not yet available (ID: NCT01000233) (U.S. National Library of Medicine (NLM), n.d.).

In the food industry PA has a Generally Recognized as Safe (GRAS) status and has been used in numerous processes: natural preservative agent in cookies (Hix et al., 1997), as additive to fish to inhibit microorganisms (Sun et al., 2020), in fruits to preserve the shelf-life and delay senescence (Du et al., 2017), in wine to prevent oxidation (Kreitman et al., 2013), in fried pork to inhibit the generation of heterocyclic aromatic amines (HAAs) (Zhang et al., 2013), to vegetables like cabbage and broccoli to reduce microorganisms (Bari et al., 2005).

In plants, PA has a wide range of functions, for example energy storage, phosphorus storage, cation source (Martínez-Domínguez et al., 2002), there is evidence that shows it is a modulator of a $K^+$ channel which regulates stomatal pore closing (Lemtiri-Chlieh et al., 2003), it is also used as antioxidant for the germinating seed (L. Bohn et al., 2008). For reviews see Raboy (2003).

Phytic acid is the main source of phosphorus in plant seeds and it is present in beans, seeds, nuts, grains. It is also found in tubers and small amounts in some fruits and vegetables like berries and green vegetables (Coulibaly et al., 2011). In

grains as well as oilseeds, phytic acid acts as a store of phosphoric acid, which is hydrolysed during sprouting which may be the reason why green leafy vegetables are virtually depleted of PA (Møllgaard, 1946).

Up to 50-80 % of the total phosphorus in plants is present in the form of phytic acid (Martínez-Domínguez et al., 2002) however the amount and distribution varies quite a lot depending on the species, for example in cereals PA constitutes 65-85 % of total P and its mainly found in the aleurone layer. Whereas roots contain only moderate amounts of phytic acid-P ranging from 21-25 % of total P, oilseeds usually have higher levels accounted up to 80 % of the total P in groundnut and gingelly (*Sesamum indicum* L.), legumes were found to contain 60-75 % of the total P (Ravindran et al., 1994).

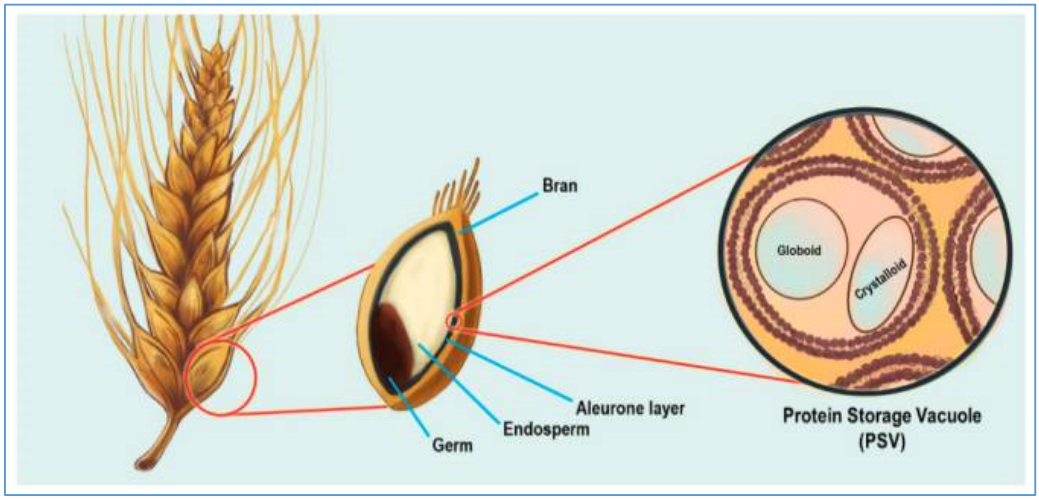



Figure 1-2 Diagram of wheat anatomy and localization of phytic acid. From Freed (2020).

In corn kernels (*Zea maize*), phytic acid-phosphorus (phytic acid-P) constitutes more that 80 % of the total P and 88 % of the phytate phosphorus is localized in the corn germ. In wheat kernels up to 87 % of the phytic acid-P is found in the aleurone layer, similarly, in rice phytic acid-P constitutes about 80 % and is located in the pericarp and aleurone (O'Dell et al., 1972). In barley 90 % of the total P is located in the aleurone layer and most of it is phytic acid (Ockenden et al., 2004). Lott (Lott et al., 2000) presented an extensive review of phytic acid content in fruits, cereals, legumes, dry seeds and fruits.

The peculiar structure of phytic acid gives the molecule a very high negative charge. Therefore it is very insoluble and tends to precipitate in the form of salts which bind strongly to important cations (Sparvoli & Cominelli, 2015). In consequence these cations are not available to be absorbed in the digestive tract of monogastric animals because they lack phytases, the molecules responsible for breaking down PA (Raboy, 2002). Previous studies on the binding of trace elements by phytic acid in vitro show that their relative affinities follow an ionotropic series $Cu^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Mg^{2+} > Fe^{2+} > Ca^{2+}$ (Maddaiah et al., 1964; Vohra et al., 1965). It has also been observed that the precipitation rate depends on whether two or more cations are present at the same time (Oberleas, 1973) as reviewed by Davies & Olpin (1979), or if certain organic acids are present during digestion. For example, citric acid, malic acid, tartaric acid are known to improve the Fe absorption (Gillooly et al., 1983; He et al., 2008).

Due to the chelating properties of phytic acid, in foods with high PA content such as wheat germ, butter beans, brown lentils and green lentils, the iron bioavailability tends to be low (Gillooly et al., 1983).

Studies have attempted to prove the enhancing properties of some organic acids on the Fe absorption, for example, human diet studies demonstrated that ingestion of whole bran reduced the iron absorption on a ratio of 0.26, (with: without bran). In meals with high iron availability, the ratio with: without bran was 0.49. The addition of ascorbic acid to the meals improved the absorption ratio but there was no improvement when compared to the dephytinized bran, thus indicating the phytic acid in bran might not be the unique cause of the iron inhibitory effect. The authors attributed the inhibitory effect to the fibre (Simpson et al., 1981).

Bosscher (2003) explored this hypothesis and concluded that dietary fibre affects the absorption of Ca, Zn and Fe depending on the type of fibre, for example, pectin, oligofructose and locust bean gum affected negatively the absorption of Fe.

## 1.4 PHYTIC ACID CONCERNS

The mineral binding capacity of the phytic acid has been in the spotlight since it contributes to mineral deficiencies in animals and humans (Harland & Morris, 1995). Which leads to another important issue: the environmental impact. Non-ruminant animals cannot digest and absorb the phosphorus in PA. Therefore all

this unused phosphorus is excreted eventually reaching water bodies and causing a major pollution problem known as eutrophication (Guttieri et al., 2004). Only from agricultural areas, the average P accumulation worldwide was 8 Tg per year (1 teragram = 1 million metric tons) from 1958 to 1998 (Bennett et al., 2001) and there is an estimation that in some areas the ruminant and pig excretion exceeds 300 kg $P_2 0_5$ per ha per year (Eeckhout & De Paepe, 1994).

To date several attempts have been made to reduce the amount of P fertilizers due to their great contribution to soil and water pollution one of such is to adjust and modify animal diets. For example, Han (1998) successfully substituted 2 % inorganic P in pigs diets of corn-soybean meal by adding a mixture of wheat middlings, phytase and citric acid and demonstrated to be as effective to maintain the plasma inorganic P concentrations, body weight and even an increase in gain-feed ratio.

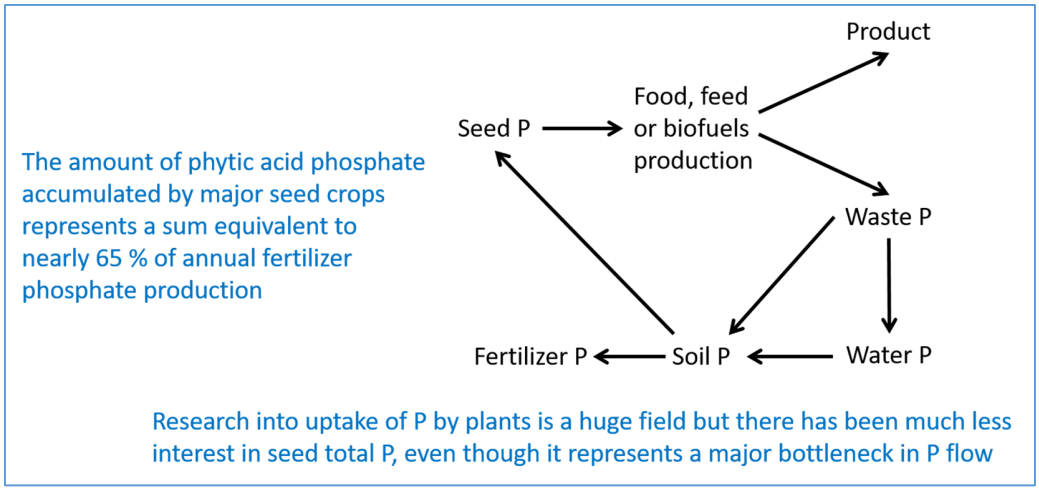



Figure 1-3 Phosphorus (P) flow through the worldwide agricultural ecosystem. Taken from (Raboy, 2020).

More recently it was estimated that nearly 35 million metric tonnes of phytic acid, containing 9.9 million tonnes of P, is combined with about 12.5 and 3.9 million metric tonnes of K and Mg respectively, to form each year over 51 million metric tonnes of phytate. Meaning that the amount of P in this phytate is nearly 65 % of fertilizer P manufactured annually worldwide and it represents a major bottleneck in P flux in agricultural ecosystems (Lott et al., 2000), Figure 1-3.

As mentioned before, monogastric animals such as swine, poultry and fish do no absorb the P in PA. The animals will therefore need phosphorus supplementation or the addition of phytase, which is costly and unlikely or rather difficult to be accessible in developing countries. Moreover, if we remember that PA chelates important cations we would have diets not only deficient in P but also very poor in vital micronutrients. In human nutrition, this is called “Hidden Hunger”.

In 2006 the WHO estimated that more than 2 billion people suffer from Hidden Hunger, meaning that they suffer from micronutrient malnutrition (MNM). The three most common forms of are iron, vitamin A and iodine deficiency. The majority of affected population live in developing countries. Iron deficiency is the most prevalent form of MNM with over 2 billion people being anaemic, just under 2 billion do not have adequate iodine nutrition and 254 million preschool-aged children are vitamin A deficient (WHO & FAO, 2006).

The Global Hunger Index (GHI) is a measure of the multidimensional nature of hunger, takes four indicators: Undernourishment, Child Wasting (percentage of

children under five years old who suffer from wasting, low weight-for-height), Child Stunting (percentage of children under five years old who suffer from stunting, low height-for-age) and Child Mortality (percentage of children who die before the age of five), taken together reflect deficiencies in calories as well as in micronutrients (von Grebmer et al., 2019).

GHI scores are calculated using a three-step process: first, values for the four component indicators are determined from the available data for each country (in %); second, the each of the indicators is given a standardized score based on thresholds set slightly above the highest country-level values observed worldwide for that indicator between 1988 and 2013. Third, the standardized scores are aggregated to calculate the GHI score for each country. Undernourishment and child mortality each contribute one-third of the GHI score, while the child undernutrition indicators—child wasting and child stunting—each contribute one-sixth of the score. This calculation results in GHI scores on a 100-point scale, where 0 is the best score (no hunger) and 100 is the worst. In practice, neither of these extremes is reached.

The GHI score shows correlations with measures of hidden hunger (Figure 1-4), such as the indicators of vitamin A, anaemia and with a proxy of diet quality for children. The correlation strength varies, for example, is moderate (0.4-0.6) for night blindness in preschool children and pregnant women, low levels of serum retinol in preschool children, and anaemia in preschool children and pregnant women. Strong correlations (>0.7) are seen for poor diet quality of

complementary foods for infants and young children. Hence, child mortality and child underweight are the two components of the GHI that make the index sensitive to variations in micronutrient deficiencies and children's dietary diversity.

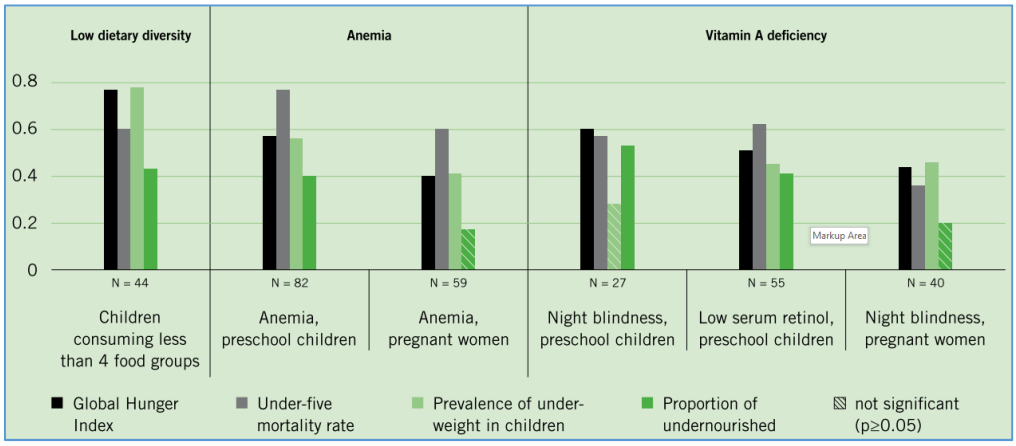



Figure 1-4 How the global hunger index correlates with measures of the hidden hunger. For footnotes refer to von Grebmer (2014).

Micronutrient deficiencies are prevalent all over the world, however the population in developing countries are far more affected and this is mainly attributed to the lack of diversity in the diet. People living on wealthier regions of the world have more access to micronutrient-rich foods such as meat, fish, poultry, eggs, milk and dairy products, as well as to a variety of fruits and vegetables whereas the population in poorer areas consume only the minimum of these sort and instead get the majority of their calorie intake from monotonous diets based on cereals, roots and tubers (WHO & FAO, 2006).

Cereals such as wheat, maize, rice, barley, millet and sorghum are the most produced cereals in the world and represent the second source of calorie intake (Figure 1-5, Figure 1-6). Although cereals are rich in micronutrients they are also the main source of phytic acid, meaning that these minerals are not bioavailable (Gillooly et al., 1983). For example, studies in barley, oats, wheat and bakery products showed they contained only IP6 in a range of 4.18 to 11.43 g $kg^{-1}$. IP6 contributed 97.2-100 % and 87.7-98.1 of the total inositol phosphates in grains and their milling products, respectively (Kasim & Edwards, 1998).

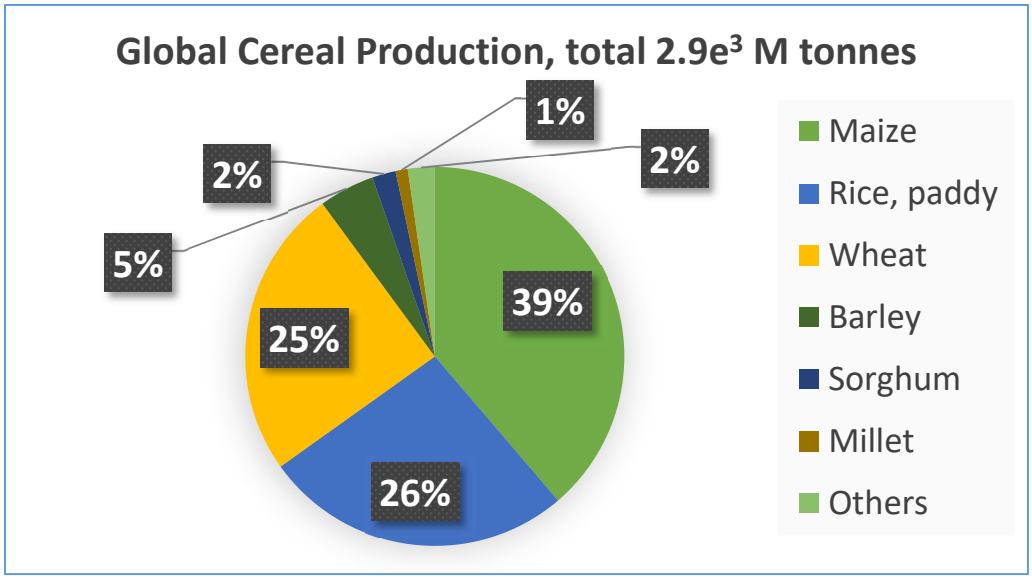



Figure 1-5 Cereals crop production (tonnes). FAOSTAT data 2018. Production data on cereals relate to crops harvested for dry grain only. Cereal crops harvested for hay or harvested green for food, feed or silage or used for grazing are therefore excluded. Aggregate, may include official, semi-official, estimated or calculated data. Other cereals: Oats, cereals not elsewhere specified, triticale, rye, mixed grain, buckwheat, fonio, canary seed, and quinoa.

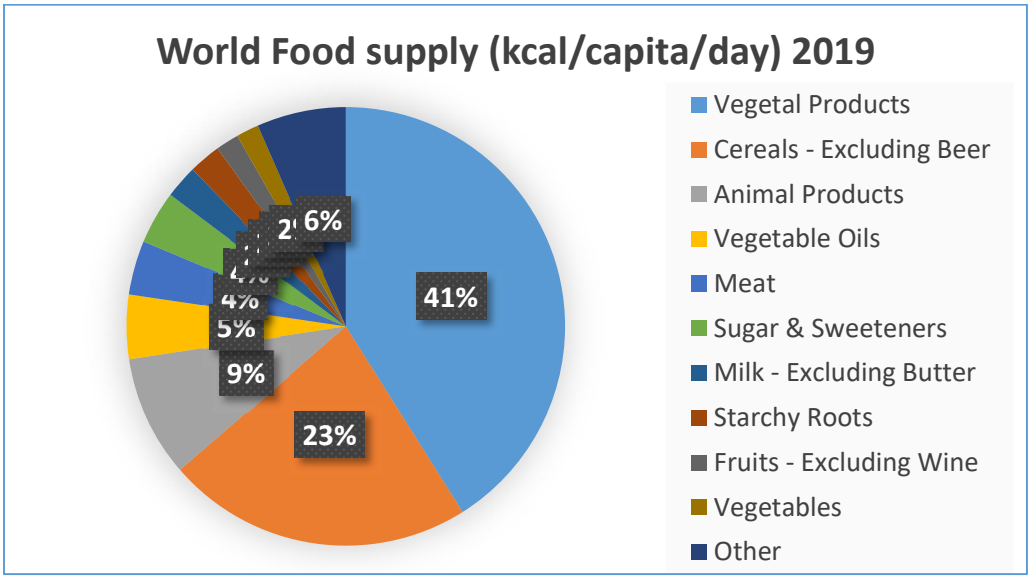



Figure 1-6 World food supply 2019. FAOSTAT data 2019.

Several strategies for control of micronutrient deficiencies have emerged, among them are: increasing the diversity of diets, fortification, supplementation and public health measures (WHO & FAO, 2006).

In the context of biofortification, removal or reduction of phytic acid from seeds represents a way to tackle the micronutrient deficiency issues. For example, a number of efforts have been made to produce and identify crop lines with low phytic acid. So far low phytic acid mutants (*lpa*) have been identified in maize (Raboy et al., 2000; J. Shi et al., 2003), rice (S. R. Larson et al., 2000), soybean (Hitz et al., 2002; Wilcox et al., 2000), wheat (Guttieri et al., 2004) and barley (S. R. Larson et al., 1998; Rasmussen & Hatzack, 1998).

Other methods include, germination or sprouting, fermentation, soaking and thermal processing. For instance, during the milling process, the endogenous

phytase gets in contact with the phytic acid thus converting it to lower phosphorylated forms with lesser adverse effects (Lehrfeld, 1989; Phillippy et al., 1988). Nonenzymatic hydrolysis generally takes place when foods are heated (e.g. autoclaving, canning) or treated with a strong acid (Q.-C. Chen & Li, 2003). Heat treatment of oats reduced the PA from 75 % to 42.6 % (Lönnerdal et al., 1989). The addition of organic acids such as tartaric, citric and lactic have proved to increase the solubility of phytate (Christensen, 1944) as cited in Møllgaard (1946).

In terms of the enzymatic treatment, studies show that the addition of a microbial phytase to diets of pigs and chickens, improves the digestibility, bioavailability and absorbance of dietary phytate P, the main downside is the cost of the enzyme supplementation (Cowieson et al., 2006; Dilger et al., 2004; Grela et al., 2020; Han et al., 1998; Kemme et al., 1997; Ketaren et al., 1993; J. W. Kim et al., 2019; Olukosi et al., 2007; Porres et al., 2006; Vallejo et al., 2018).

In wheat varieties, heating treatment reduced PA content 27-32 %, soaking decreased it 22.5-25.1 % and germination treatment 37-40 % (Masud et al., 2007). Although these methods are easy to apply and relatively costless, but with a big downside, the fact that other minerals such as Zn and Fe are lost. For example the studies of Afify (2011), when soaking sorghum, they observed Fe losses of 28.16-40.06 % and Zinc content decreased 13.78-26.69 % contrasting with the raw grain. Germination also lead to a Fe loss of 38.45-39.18 and Zinc of 21.80-31.27 % compared to the raw grain. The author also report increases on the *in vitro* bioavailability of Fe and Zn after the soaking and germination process.

Baking and fermentation processes reduced in 58.8 % the PA in bread compared to the unbaked and unfermented wheat flour. The same observation was for rye bread where there was a reduction of 29 % of the PA bread versus unprocessed rye flour, (Dost & Tokul, 2006).

Autoclaving has shown little effect on the phytate solubility. Furthermore, it does not change the amount of phytate but it can change the level of phosphorylation of the inositol phosphates. A study that involved autoclaving soybean flakes for 2 h converted at least 10 % of the inositol hexaphosphate to the pentaphosphate. After 4 h, the tetraphosphate was detected and the amount of the pentaphosphate was almost equal to that of the original hexaphosphate (de Boland et al., 1975).

## 1.5 PHYTIC ACID AND OTHER INOSITOL PHOSPHATES IN ANIMAL AND HUMAN NUTRITION

As discussed in the previous section, phytic acid (PA) forms strong complexes with metals such as iron, aluminium, copper, calcium and magnesium (Vohra et al., 1965). In grains used for human consumption and for feeding farm animals 75 to 85 % of the total phosphorus is found as phytic acid (Møllgaard, 1946).

The small intestine presents a favourable environment (pH 4.6-6.9) for the formation of pentacalcium phytate (Hoff-Jørgensen, 1944) as cited in Møllgaard (1946). Leading to poor absorbance of calcium. At pH 4.0 or above mixtures with Ca:P ratios (840:115 mg) and P present as phytate, precipitate quite rapidly (Hill &

Tyler, 1954). Diets high in Ca:P ratios and large amounts of phytate have been shown to give rickets in rats (Bruce & Callow, 1934; Krieger & Steenbock, 1940; Lowe & Steenbock, 1936).

Studies in rats demonstrated the huge effect that PA has over Zn bioavailability. Growth rates, plasma Zn concentrations, hair Zn concentrations were affected and produced greying of the coat at values of phytate:Zn ratios of 15:1, 10:1, 15:1 and 15:1 respectively (Davies & Olpin, 1979).

Phytic acid hydrolysates can also exert an effect on mineral bioavailability. Studies in rats, demonstrated that the higher the degree of phosphorylation, the higher the inhibition effect over Zn and Ca. Liver Zn uptake from a solution of IP6 was only 5 %, 19 % from IP5, 28 % from IP4 and 29 % from IP3 whereas for the control solution ($ZnCl_2$) the uptake was 31 %. Non-absorbed calcium was 17 %, 1.4 %, 0.5 %, 0.5 % and 0.5 % of the given dose of $^{45}Ca$, respectively. Feeding studies with Japanese quail, IP5 and IP6 had similar detrimental effects on the bioavailability of zinc, while IP3 and IP4 had no effect (Tao et al., 1986). For iron absorption there were similar results (Sandberg et al., 1989).

PA also inhibits magnesium absorption in a dose dependent way. Studies with human meals demonstrated that the fraction apparent magnesium absorption decreased from 32.5 % to 13 % when 1.49 mmol of PA were added to white bread and 32.2 % to 24.0 % with 0.75 mmol PA (T. Bohn et al., 2004).

Numerous studies have attempted to estimate the human PA intake from diets; some of them are summarized on Table 1-2. The average American consumes around 750 mg of phytic acid per day with a range of (300-1300 mg per day) Harland and Peterson (1981), cited by (Fordyce et al., 1987). Other studies in American women report an intake of PA of 395 mg $day^{-1}$ (Murphy & Calloway, 1986). The impact that PA has on the bioavailability of minerals varies widely according to the diet composition and particular characteristics, it is therefore a complex variable. For example, a vegetarian diet contains about 2575 mg of PA and 11.2 mg of zinc per day, contrasting with the non-vegetarian menu, which contains 290 mg of phytic acid and 11.4 mg of zinc daily (Ellis et al., 1982; Oberleas & Harland, 1981) as cited by Fordyce (1987).

Table 1-2 Phytate daily intakes of diverse populations in the world. From Reddy (2002).

| Country | Groups | Mean phytate (mg day$^{-1}$) | Range/SD |
|---|---|---|---|
| US | Non-lactating young women (18-24 yrs) | 395 | ± 14 |
| US | Lacto-ovo-vegetarian Trappist monks (1977) | 4569 | 615-5770 |
| | Lacto-ovo vegetarian Trappist monks (1987) | 972 | 58-3186 |
| US | College students (19-35) | 1293 | 198-3098 |
| US | Omnivorous females | 631 | 585-734 |
| | Omnivorous males | 746 | 714-781 |
| Canada | Preschool girls (4-5 yrs) | 250 | 132-318 |
| | Preschool boys (4-5 yrs) | 320 | 203-463 |
| Mexico | Toddlers (18-30 months) | 1666 | ± 650 |
| Guatemala | Pregnant women (15-37 yrs) | 2254 | 877-4708 |
| UK | Students and faculty staff | 670 | 500-840 |
| UK | - | - | 600-800 |
| UK | Infants (1-18 months) | - | 0-200 |
| Sweden | - | 180 | - |
| Italy | - | 219 | 112-1367 |
| Egypt | Toddlers (18-30 months) | 796 | ± 248 |
| Taiwan | Graduate students and faculty members (20-60 yrs) | 780 | ± 260 |
| Nigeria | - | 2100 | 2000-2200 |
| Malawi | Preschool girls (4-6 yrs) | 1675 | 1621-2161 |
| | Preschool boys (4-6 yrs) | 2010 | 1857-2161 |
| Ghana | Omnivorous children | 578 | ± 161 |
| Papua New Guinea | Children | 569 | ± 561 |
| Kenya | Toddlers (18-30 months) | 1066 | ± 324 |
| India | Faculty families | 670 | 596-742 |
| | Adolescents (10-19 yrs) | 1565 | 1350-1780 |
| | Adults (20-45 yrs) | 2030 | 1560-2500 |
| | Adults (>45 yrs) | 168 | 1290-2080 |
| India | Lacto-vegetarian young women (16-20 yrs) | 840 | - |
| | Non-vegetarian young women (16-20 yrs) | 848 | - |
| Gambia | Infants (1-17 months) | - | 10-560 |

SD, standard deviation

The PA:Zn values can be used as indicator of the availability of Zn in diets with high content in phytate (Davies & Olpin, 1979), although some authors have reported the PAxCa:Zn to be a better predictor (Bindra et al., 1986; Davies & Olpin, 1979). A population with a vegetarian diet besides the high content of PA also has a high calcium intake, from for example, the calcium sulphate in tofu, wholegrain cereals and legumes, which contributes to the inhibitory effect of PA (Fordyce et al., 1987). In contrast, a study with pigs reported that the supplementation with calcium decreases the degradation of PA in the colon but not in the stomach and small intestine, suggesting that the minerals can still be absorbed due to an intestinal phytase or alkaline phosphatase activity or even microbial activity (Sandberg et al., 1993).

Based on evidence, diets with Ca x PA:Zn ratio greater than 150 mmol/1000 kcal are expected to reduce the zinc bioavailability. The predicted fractional absorption (%) of diets with a PA:Zn ratio greater than 15 are classified as poorly available (Type C), between 5-15 moderately available (Type B) and lower than 5 highly available (Type A), Figure 1-7. The efficiency of utilization can go from 50 % with type A diets, 30 % on type B and 15 % on type C diets. Table 1-3 presents some criteria for categorizing diets according to the potential availability of zinc content (World Health Organization, 1996). For instance, in American omnivorous diets the PA:Zn and PAxCa:Zn were less than 10 and 200 respectively, suggesting the studied population is not at risk of developing Zn deficiencies (Ellis et al., 1987).

The IZiNCG equation predicts that in diets with PA:Zn molar ratios of 5-18 the zinc absorption is 31 % whereas for ratios above 18 the absorption falls to 23 %. The IZiNCG reviewed the parameters used to estimate the physiologic requirements for absorbed zinc during childhood by age group and sex, and during pregnancy and lactation, which had been previously developed by the WHO and the US FNB/IOM (US Food and Nutrition Board/Institute of Medicine Standing Committee on the Evaluation of Dietary Reference Intakes). In order to produce a new model, IZiNCG took into consideration the methodology used to measure absorption, the types of diets and subjects from which data were derived, as well as the models used to summarize these data. Therefore the dietary requirements account for the effects of phytate on total zinc intake requirements, whereas the FNB/IOM dietary requirements did not consider the effects of phytate (Brown et al., 2004).

Later, Miller (L. V. Miller et al., 2007) developed a complex mathematical model of zinc absorption, as a function of dietary zinc and phytate, this equation was used in a more recent model presented by Wessells & Brown (2012) to estimate the global prevalence of zinc deficiency. The authors ranked countries using a combined model using the IZiNCG nutrient composition database, the Miller equation to predict zinc absorption and an assumed coefficient of variation in zinc intake (25 %).

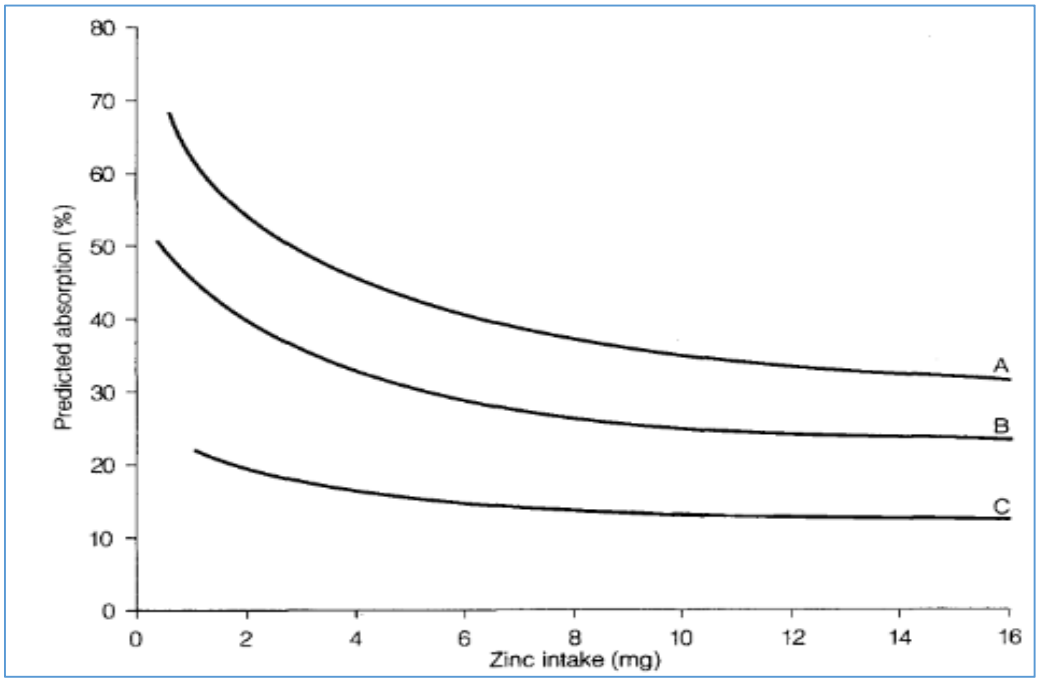



Figure 1-7 Predicted fractional absorption (%) by 65-kg men of dietary zinc derived from diets in which zinc is highly available (category A), moderately available (category B) or poorly available (category C). From World Health Organisation (1996).

Taking all together the newest models allow to identify the regions where the prevalence of zinc deficiency is greater and therefore target programs and interventions to assess this issues. South Asia presented the highest percentage of population with inadequate zinc intake (30 %), followed by Sub-Saharan Africa (26 %) and the East and Southeast Asia and Pacific region (22 %), globally, it was estimated that 17 % of the population are at risk of inadequate zinc intake.

Table 1-3 Provisional criteria for categorizing diets according to the potential availability of their zinc.

| Nominal category | Principal dietary characteristics |
|---|---|
| A. High availability | i. Refined diets low in cereal fibre, low in phytic acid content and with phytate:zinc (molar) ratio <5; adequate protein sources, such as meats, fish.<br>ii. Includes semisynthetic formula diets based on animal protein. |
| B. Moderate availability | i. Mixed diets containing animal or fish protein.<br>ii. Lacto-ovo, ovovegetarian or vegan diets not based primarily on unrefined cereal grains or high- extraction-rate flours.<br>iii. Phytate:zinc molar ratio of total diet within the range 5-15 or not exceeding 10 if more than 50 % of the energy intake is accounted for by un-fermented, unrefined cereal grains and flours while the diet is fortified with inorganic calcium salts ( > 1 g $Ca^{2+}$/day).<br>iv. Availability of zinc in category B foods improves when the diet includes animal or protein sources or milks. |
| C. Low availability | i. Diets high in unrefined, unfermented and un- germinated cereal grain, [a] especially when fortified with inorganic calcium salts and when intake of animal protein is negligible.<br>ii. Phytate:zinc molar ratio of total diet exceeding 15.[b]<br>iii. High-phytate soya-protein products constitute the primary protein source.<br>iv. Diets in which, singly or collectively, approximately 50 % of the energy intake is accounted for by the following high-phytate foods: high-extraction-rate (90 % +) wheat, rice, maize grains and flours, oatmeal, millet; chapatti flours and "tanok"*; sorghum; cowpeas; pigeon peas; grams; kidney beans; blackeye beans; groundnut flours.<br>v. High intakes of inorganic calcium salts (> 1 g $Ca^{2+}$ /day), either as supplements or as adventitious contaminants (e.g. from calcareous geophagia), potentiate the inhibitory effects of category C diets; low intakes of animal protein ex-acerbate these effects. |

[a] Germination of many of such grains or fermentation (e.g. leavening) of many flours can reduce antagonistic potency; the diet should then be reallocated to category B.

[b] Vegetable diets with phytate:zinc ratios exceeding 30 are not unknown; for such diets, an assumption of 10 % availability of zinc or less may be justified, especially if the protein intake is low and/or that of calcium salts is excessive, e.g. > 1.5 g $Ca^{2+}$ per day. (World Health Organization, 1996).

*Tanok is an Iranian bread almost identical to chapatti.

Several studies have reported phytic acid intake in different diets, some examples are given here onwards. In African diets, the mean PA intake was 2770 mg capita$^{-1}$ day$^{-1}$, range (1004 to 4769 mg capita$^{-1}$ day$^{-1}$) and the Phytate:Zn molar ratios

ranged from 7.2 to 37.7 with mean and national median ratios of 22.6 and 19.9, respectively. The main PA supply was from cereals with 68 %, followed by pulses and beans 17 % and roots and tubers 7 % (Joy et al., 2014).

In preschool children from Ontario, Canada, the median millimolar ratio of PA:Zn was 5.3 with no differences between boys and girls. Whereas the median intakes of phytate (M, 399; F, 333 mg $d^{-1}$) and median millimolar CaxPA:Zn ratios (M, 102.1; F, 72. 3) were higher for boy and for girls (Gibson et al., 1991). In 2-year-old Mexican children the mean PA intake was 545 ± 190.1 mg $day^{-1}$ with an average PA:Zn molar ratio of 7 (Cantoral et al., 2015).

South Korean diets contain 1676.6 mg $day^{-1}$ of phytate. The ratio PA:Zn was 15.9 mol $day^{-1}$, and PAxCa:Zn was 168.9 mmol $day^{-1}$. Cereals and grain products were the main source of phytate (46 %) followed by seasonings (garlic), fruits and legumes and their products. Rice was the major food source of phytate (39 %) (Kwun & Kwon, 2000).

Research in Chinese rural and urban diets showed a median daily intake of PA of 1186 mg $day^{-1}$ with significant differences between the rural and urban populations (1342 and 781 mg $day^{-1}$, respectively). The median molar ratio PA:Zn was 11.1, the rural areas had significantly greater ratios when compared to the urban areas, 12.5 and 7.3 respectively (G. Ma et al., 2007).

The median daily intake of PA in British population was 496, 615, 809 and 629 mg $day^{-1}$ in children, adolescents, adults and elderly respectively. The ratios PA:Zn

were 11.8, 10.4, 9.7 and 8.7 respectively. The main sources of phytate in this population are cereal and cereal products, vegetables, potatoes and savoury snacks, hot drinks, commercial toddlers' foods and drinks, chocolate, soups, fruits and nuts (Amirabdollahian & Ash, 2010).

In Trappist monks, it was observed that the intakes of phytate-containing foods had decreased from 4569 to 972 mg $day^{-1}$; intake of dietary zinc had increased from 7.4 to 9.7 mg $day^{-1}$; and the PA:Zn molar ratio had decreased from 67 to 14 for the years 1977 and 1987, respectively (Harland et al., 1988).

Asian immigrants in Canada consuming predominantly lacto-ovo vegetarian diets based on chapatti and beans were compared with 30 omnivorous diets. The mean PA daily intake was 1487 ± 791 mg. The median PA:Zn molar ratio for Punjabi diets was 17.7 (range 5-50) and median PAxCa:Zn molar ratio was 475.4 (range 48-1150), notably higher when compared to the omnivorous diets (8.3 and 130.9 respectively) (Bindra et al., 1986).

## 1.6 PHYTIC ACID IN FOODS AND FOODSTUFFS

Phytic acid is widely distributed in plants. It constitutes the main form the plant stores phosphorus and minerals, during germination the molecule is hydrolysed. Phytic acid (IP6) is predominantly present in unprocessed food, processing can degrade IP6 to a lower phosphorylated forms. The lower phosphorylated forms contain fewer phosphate groups and therefore the highly negative charge of the

molecule is reduced leading to fewer cations being chelated. This is why the lower phosphorylated forms are not considered to have a great impact in the bioavailability of minerals and consequently they do not contribute to mineral deficiencies in monogastric animals.

The concentrations present in food vary extensively, cereals and legumes are the main reservoir of PA constituting up to 60-80 % of the total phosphorus. Nuts content is around 0.1-9.4 % contrary to green leafy vegetables such as lettuce, onions, mushrooms, celery, spinach and some fruits like citrus, apples, bananas and prunes are practically devoid of the antinutrient. Other vegetables like potatoes, sweet potatoes, artichokes and fruits like blackberries, strawberries and figs contain moderate amounts of phytate (Oberleas, 1973). Oilseeds can contain between 1-5.4 %, dry weight basis. To illustrate, Table 1-4 was prepared taking a few examples of unprocessed samples (except for the flours) from previous reports. For full studies consult: (Adeyeye et al., 2000; Alkarawi & Zotz, 2014a; Eeckhout & De Paepe, 1994; Gibson, 2012a; Ravindran et al., 1994; Schlemmer et al., 2009).

Table 1-4 Phytic acid content of various uncooked foods. Units of measure varied depending on the original study. Weight is in dry weight basis (DWB) unless specified.

| Sample | Phytic acid | Reference |
|---|---|---|
| **Cereals** | | |
| Maize flour 95 % extraction | 7.92 mg $g^{-1}$ | (Gibson, 2012b; Gibson & Ferguson, 2008) |
| Rice raw | 2.11 mg $g^{-1}$ | (Gibson, 2012b; Gibson & Ferguson, 2008) |
| Sorghum flour | 4.46 mg $g^{-1}$ | (Gibson, 2012b; Gibson & Ferguson, 2008) |
| Wheat bran | 21 - 73 mg $g^{-1}$ | (Schlemmer et al., 2009) |
| Wheat germ | 11.4 - 39.1 mg $g^{-1}$ | (Schlemmer et al., 2009) |
| Barley | 3.8 - 11.6 mg $g^{-1}$ | (Schlemmer et al., 2009) |
| Oat | 4.2 - 11.6 mg $g^{-1}$ | (Schlemmer et al., 2009) |
| Millet | 1.8 - 16.7 mg $g^{-1}$ | (Schlemmer et al., 2009) |
| Rye | 4.52 ± 0.22 mg $g^{-1}$ | (García-Estepa et al., 1999) |
| **Legumes** | | |
| Mung beans | 3.87 ± 20.46 mg $g^{-1}$ | (Q.-C. Chen, 2004) |
| Navy beans | 8.32 ± 244.2 mg $g^{-1}$ | (Q.-C. Chen, 2004) |
| Pinto beans | 7.97 ± 283.8 mg $g^{-1}$ | (Q.-C. Chen, 2004) |
| Red kidney beans | 6.07 ± 118.8 mg $g^{-1}$ | (Q.-C. Chen, 2004) |
| Cowpeas | 9.9 mg $g^{-1}$ | (Ravindran et al., 1994) |
| Lentils | 7.1 mg $g^{-1}$ | (Ravindran et al., 1994) |
| **Roots and tubers** | | |
| Cassava | 1.4 mg $g^{-1}$ | (Ravindran et al., 1994) |
| Sweet potato | 1.8 mg $g^{-1}$ | (Ravindran et al., 1994) |
| Taro | 3.2 mg $g^{-1}$ | (Ravindran et al., 1994) |
| Yam | 1.1 mg $g^{-1}$ | (Ravindran et al., 1994) |
| **Oilseeds** | | |
| Linseed | 21.5 - 36.9 mg $g^{-1}$ | (Schlemmer et al., 2009) |
| Rapeseed | 25 mg $g^{-1}$ | (Schlemmer et al., 2009) |
| Sesame seed | 14.4 - 53.6 mg $g^{-1}$ | (Schlemmer et al., 2009) |
| Groundnut | 14.2 mg $g^{-1}$ | (Ravindran et al., 1994) |
| **Vegetables** | | |
| Spinach | 0.7 mg $g^{-1}$ | (Ravindran et al., 1994) |
| Sweet potato leaves | 0.7 mg $g^{-1}$ | (Ravindran et al., 1994) |
| Cucurbita maxima | 92.3 mg $g^{-1}$ (FW) | (Gupta et al., 2005) |
| *Amaranthus tricolor* | 19.5 mg $g^{-1}$ (FW) | (Gupta et al., 2005) |
| *Cocculus hirsutus* | 44 mg $g^{-1}$ (FW) | (Gupta et al., 2005) |
| *Polygala erioptera* | 33.8 mg $g^{-1}$ (FW) | (Gupta et al., 2005) |
| Turnip | 0.198 mg $g^{-1}$ | (Harland & Morris, 1995) |
| Carrot | 0.40 mg $g^{-1}$ | (Harland & Morris, 1995) |
| Celery | 0.132 mg $g^{-1}$ | (Harland & Morris, 1995) |

## 1.7 PHYTIC ACID IN WHEAT: SOME STUDIES

Several studies are available in the literature that have measured phytic acid concentrations in wheat samples, some examples of them are listed on Table 1-5 below.

Table 1-5 Phytic acid concentration on varied wheat sources.

| Sample | Mean PA ± SD | Range | Reference |
|---|---|---|---|
| Wheat flour F20* | 10.63 ± 0.09 mg $g^{-1}$ | - | (Kashlan et al., 1990) |
| Wheat flour F30* | 7.98 ± 0.03 mg $g^{-1}$ | - | |
| Wheat flour F40* | 2.38 ± 0.08 mg $g^{-1}$ | - | |
| Tanouri flour | 7.38 ± 0.05 mg $g^{-1}$ (dry basis) | - | |
| Khoboz Abyed Arabi | 0.33 ± 0.05 mg $g^{-1}$ | - | (Kashlan et al., 1990) |
| Khoboz Asmer Arabi | 6.96 ± 0.02 mg $g^{-1}$ | - | |
| Khoboz Asmer Belnokhalla | 10.45 ± 0.05 mg $g^{-1}$ | - | |
| Khoboz Tanouri bread | 6.25 ± 0.02 mg $g^{-1}$ (dry basis) | - | |
| Refined wheat flour | 3.77 mg $g^{-1}$ hand-made refined flours<br>2.96 mg $g^{-1}$ for factory-made refined flours | Range of 2-4 (mg $g^{-1}$) | (Febles et al., 2002) |
| Whole wheat flour | 8.50 mg $g^{-1}$ | Range 6-10 (mg $g^{-1}$) | |
| Gofio (roasted wheat flour) | 6.27 mg $g^{-1}$ | - | (Febles et al., 2000) |
| Infant flours | 24.6 mg $g^{-1}$ | - | (Febles et al., 2001) |
| Coarse bran | 53.85 mg $g^{-1}$ | - | (Wu et al., 2010) |
| Shorts | 28.45 mg $g^{-1}$ | - | |
| Smaller bran sizes | 54 to 5.09 mg $g^{-1}$ | - | |
| 15 genotypes of *T. aestivum* | - | 14.21 to 17.86 mg $g^{-1}$ | (Branković, Dragičević, Dodig, Knežević, et al., 2015) |
| Indian bread wheat | - | 23.9 mg $g^{-1}$ | (Yenagi & Basarkar, 2008) |
| Indian durum wheat | | 19.3 mg $g^{-1}$ | |
| 66 mutants and cultivars of bread wheat | 15 mg $g^{-1}$ | 9.8 to 21.7 mg $g^{-1}$ | (Khan et al., 2007) |
| 100 advanced breeding lines of wheat | 9.59 mg $g^{-1}$ of seed weight | 4.97 to 15.02 mg $g^{-1}$. | (Shitre et al., 2015) |
| Wheat bran | 0.612 mg $g^{-1}$ | 4.2-6.1 % | (Anjum et al., 2002)* |
| Whole wheat flour | 0.223 mg $g^{-1}$ | 1.2-2.2 % | |
| Straight grade flour | 0.024 mg $g^{-1}$ | 0.2-0.5 % | |

*Values were converted using $1hL^{-1} = 10000$ kg; * flour extraction rate; PA, phytic acid; SD, standard deviation.

## 1.8 SOURCES OF WHEAT DIVERSITY

Using existing diversity is important to identify lines with altered PA levels that could be used in breeding programs to develop novel low PA varieties.

Genetic diversity is a key factor to find and develop nutritionally improved germplasm. Modern elite wheat cultivars have lost most of the genetic diversity once present in landrace cultivars. Further losses can be prevented by the introgression of material from landraces (Reif et al., 2005; Smale et al., 2002). A. E. Watkins gathered a collection of around 7000 bread wheat accessions predominantly from Asia and Europe (Watkins, 1928), Figure 1-8. Nowadays the set has 826 accessions and is called Watkins collection. Exploring and exploiting the genetic diversity present in the Watkins collection can help in the development of more resilient and even more nutritious wheat breeding resources (Wingen et al., 2014). In terms of phytic acid, much is left to uncover. Discovery and mapping PA biosynthetic genes in wheat remains a big area to explore.

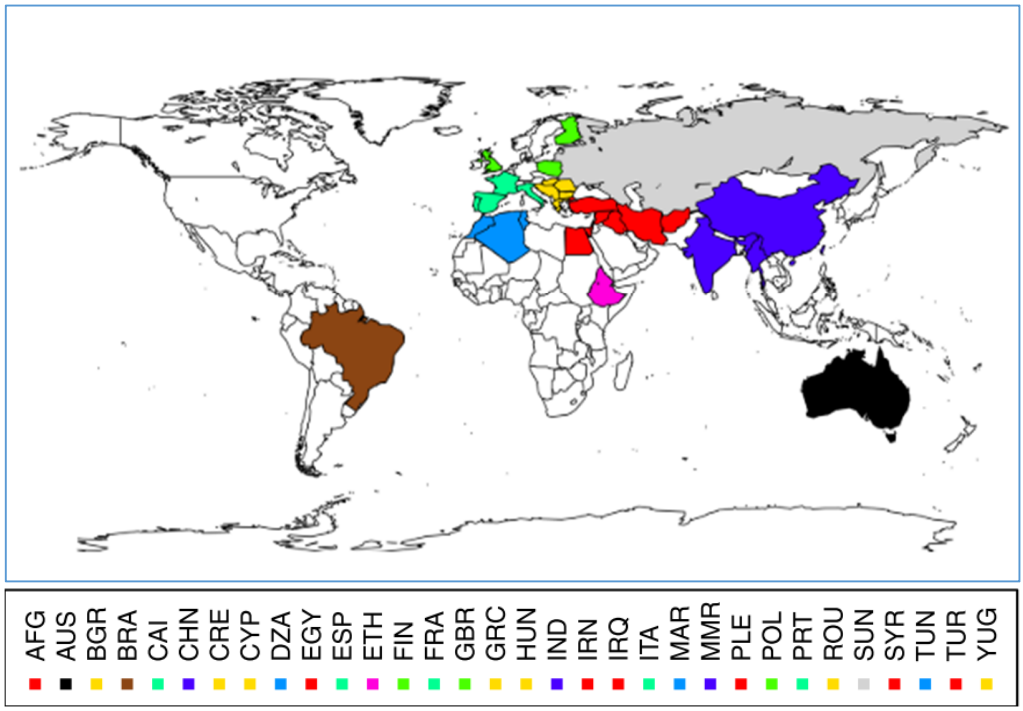



Figure 1-8 A. E Watkins: Countries of Origin-Regions. Countries from which the landrace cultivars were acquired are coloured. Colours are organised in geographic regions. At the bottom is shown the colour code of the countries, according to geographic regions and the abbreviation of the country name. AFG, Afghanistan; AUS, Australia; BGR, Bulgaria; BRA, Brazil; CAI, Canary Islands; CHN, China; CRE, Crete; CYP, Cyprus, DZA, Algeria; EGY, Egypt; ESP, Spain; ETH, Ethiopia; FIN, Finland; FRA, France; GBR, UK; GRC, Greece; HUN, Hungary; IND, India; IRN, Iran; IRQ, Iraq; ITA, Italy; MAR, Morocco; MMR, Myanmar; PLE, Israel; POL, Poland; PRT, Portugal; ROU, Romania; SUN, USSR; SYR, Syria; TUN, Tunisia; TUNR, Turkey; YUG, Yugoslavia. From: (Wingen et al., 2014).

## 1.9 RELATIONSHIP BETWEEN THE NUTRIENT STATUS OF THE PLANT AND PHYTATE LEVELS

Early studies have demonstrated a positive correlation between available soil phosphorus and seed total phosphorus in oats (W. E. Larson et al., 1952; Moore et al., 1957) (reviewed in Miller et al., 1980) and wheat (Bains, 1949; Boatwright & Haas, 1961; Srivastava et al., 1955) (reviewed in Miller et al., 1980). Moreover,

available soil P has been positively correlated with phytic acid in wheat (Bains, 1949; Srivastava et al., 1955) (reviewed in Miller et al., 1980) and similar correlations have been reported for other cereals like oats, barley, soybeans and beans (Lolas et al., 1976). Later Miller (1980) concluded that there was a positive relationship between the groat phytic acid and the soil available phosphorus in seven cultivars of oats. However, the author noted that the amount of groat phytic acid synthesis with increasing available soil P was not significantly different among cultivars within each year but was significantly different between years, therefore suggesting a strong influence of the environment.

Uppström & Svensson (1980) reported (private communication with R. Ohlson), that phytic acid content in rapeseed grown in a field, which had not been fertilized in 20 years, was reduced. Their results implied that the phytic acid content was mostly influenced by environmental factor such as the availability of phosphorus in the soil.

More evidence supporting these statements was reported by Rivera-Reyes (2009), the authors linked phosphorus and nitrogen fertilization, with the PA content in oat seeds. In soybean, Raboy & Dickinson (1984, 1987) arrived to the same conclusions correlating phosphorus and zinc fertiliser levels with the accumulation of phytic acid in seeds. Moreover, it was found the leaf P concentrations were closely related to those of P and PA in seed. In wheat, Batten &Lott (1986) examined the accumulation of phytate in kernels grown in low-P nutrient solution, the authors found the low-P grown kernels began accumulating phytate

phosphorus at 31 days after pollination (DAP) contrary to the control grains which accumulated rapidly starting at 14 DAP. They also reported the globoids in the aleurone layer is dominated by P, K and Mg and observed an increase in the K:P ratio in low-P grown grains. Likewise, Eeckhout & De Paepe (1994) found that P content is positively correlated with phytic acid-P content in wheat grain and wheat by products and maize and maize by products.

Rice hydroponic experiments with different levels of exogenous phosphorus, support previous findings. Increased external P supply increased phytic acid concentration in grain (g $kg^{-1}$), but it did not increase the amount of grain PA accumulation on per grain basis (mg $grain^{-1}$), additionally the content and bioavailability of Zn and Fe were affected in a negative fashion (Su et al., 2018). Wheat *lpa* experiments suggested the environmental effect over grain PA and P concentrations is just as great as the genotype alone (Guttieri et al., 2006a) suggesting that the soli P might have an effect over the P distribution (phytic acid-P to Pi) in the *lpa* genotype (Guttieri et al., 2006c). On the other hand, a recent study found that phytic acid-P in seed of an *lpa* and wild type soybean cultivars was not impacted by the rates of P fertilization, however the inorganic P was higher in *lpa* lines grown with more P supply (Taliman et al., 2019).

Instead of reducing the PA concentration, zinc soil fertilization has been proposed as a way to increase Zn accumulation in crops and by this improve the negative effect of PA over Zn bioavailability, in this context, several trials have been successful. For example, wheat leaves and grain showed increased accumulation

of zinc as the Zn fertilization was increased. Phytic acid concentration in grain decreased with increased rates of Zn and similarly, the ratio PA:Zn was reduced from over 30 in treatment zero to below 10 in the highest Zn treatment (Z. Wang et al., 2015)

In other experiments, zinc and nitrogen fertilization were found to affect negatively the seed phosphorus and phytic acid concentration in chickpeas. In seed, total phosphorus was positively correlated with phytic acid-P, PA:Zn millimolar ratio and PA contrary to Zn which displayed a negative correlation with phytic acid-P, PA:Zn millimolar ratio and PA (Kaya et al., 2009).

It has been found in rice studies that phytic acid has a significant positive correlation with minerals such as Fe, Zn, Cu and Mn, moreover PA is highly correlated with IP5, the inorganic P and especially with the total P, confirming most of the P in grain is stored as PA (Stangoulis et al., 2007). Selenium was the only element that had a correlation between leaf and grain in rice (Norton et al., 2010).

Further research in *Brassica napus* reaffirmed the last statement, showing highly correlated phosphorus and phytic acid in both seed and leaf (Figure 1-9), there was no correlation between phytate in seeds and phosphorus in leaf (Zhao et al., 2007).

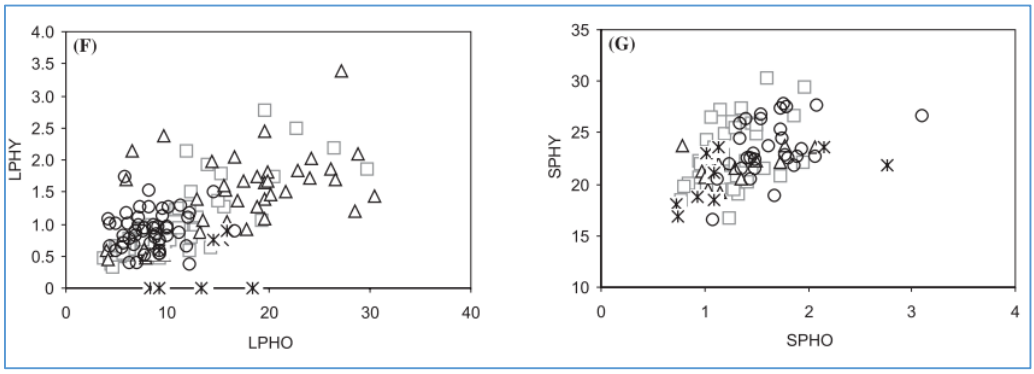



Figure 1-9 Correlation between phosphorus and phytic acid. Right (F), leaf phosphorus (LPHO) and leaf phytic acid (LPHY); left (G), seed phosphorus (SPHO) and seed phytic acid (SPHY) of 160 *Brassica rapa* accessions. Data are in mg $g^{-1}$ dry mass. The different symbols refer to different subgroups of the population analysed: □, S1; Δ, S2; x, S3; ○, S4. From (Zhao et al., 2007).

The precise role of phytic acid in leaves is still unknown. It has been documented in leaves that the proportions of phytic acid-P tend to decrease with the increasing P status of the plant, contrary to the generally observed positive correlation (Alkarawi & Zotz, 2014b). The same authors reported on a previous study, the negative correlation between the proportion of phytic acid (phytic acid-P/total P, expressed as percent) and the total P in *Manihot esculenta* leaves.

## 1.10 PHYTIC ACID REDUCTION IN PLANTS, THE GENETIC APPROACH

To address the numerous issues related to phytic acid such as reducing the amount of phosphorus sequestered by the plant, decrease the phosphorus release to the environment via the animal excretes and improving the phosphorus digestibility and therefore better intake of micronutrients in monogastric animals

including humans, a number of scientists have advocated their research into altering the phytate content of crops.

Efforts into understanding the phytic acid biosynthetic pathway have led to the discovery of a number of low phytic acid lines. Even though this topic goes beyond the scope of this study, it is important to mention the progress made so far in this area. The following paragraphs describe some genes that have been studied and characterised as potential candidates for genetic engineering plants with low phytic acid content.

In rice, Yoshida (1999) studied the I(1)P synthase that catalyses the first step of inositol metabolism, as a potential candidate to engineer low phytic acid lines. A year later, the first *lpa1* mutation was reported (S. R. Larson et al., 2000), the rice mutant exhibited 39 % of the total phosphorus as phytic acid compared to 71 % of the wild type and the Pi (inorganic phosphorus) represented 32 % of total P compared with 5 % of the wild type. The responsible gene was cloned and identified as *OsLpa1;* and was homologous to a 2-phosphoglycerate kinase (2-PGK) (S. I. Kim, Andaya, Goyal, et al., 2008). There were two more mutants reported, with reductions of 73 % and 43 % of PA compared to the wild type, the decrease was accompanied by an increase in Pi but without the accumulation inositol intermediates, similar to the *lpa3* in maize. This gene was designated as *OsMIK* based on the homology to the maize MIK gene (S. I. Kim, Andaya, Newman, et al., 2008). The gene *OsMRP5,* is the rice orthologue of the maize *ZmMRP4.*

Mutations on this gene produced rice lines with reductions of phytic acid-P of ~23 %, >90 % and ~90 % (Xu et al., 2009).

One of the biggest difficulties with the *lpa* crops is that many of the lines produced induced undesirable agronomic characteristic such as low yield, poor germination, low viability, and low seed weight among others. Nevertheless, some studies have demonstrated that it is possible to obtain seeds with significant reductions in phytic acid without affecting agronomical traits (Campion et al., 2009; J. Shi et al., 2007; Spear & Fehr, 2007). More recently, a SULTR-like phosphorus distribution transporter (SPDT) was reported. In the knockout lines, the distribution of phosphorus was altered; phytic acid levels were decreased in seeds but increased in leaves. Total phosphorus and phytate in the brown de-husked rice were 20–30 % lower in the knockout lines and the yield, seed germination and seedling vigour were not affected. It was concluded from this outcomes that the SPDT functions as a switch node for the allocation of phosphorus (Yamaji et al., 2017). Other examples are the KBNT *lpa1-1* that has 90 % yield of the standard Arkansas rice cultivars (Rutger, Raboy, et al., 2004) and Goldhull Low Phytic Acid (GLPA) rice (Rutger, Bryant, et al., 2004).

A small loss of yield compared to the wild type is practically unavoidable but these new seeds are promising, since they can contribute to mitigate some of the adverse effects of phytic acid as well as potential money saving from P fertilizers and supplementation to animal foodstuffs.

In soybean, the mutation *lr33* was described by Hitz (2002). The mutation was localised in the *myo*-inositol 1-phopshtate gene and produced a phenotype with lower content in raffinosaccharide and phytic acid (63 % less *myo*-inositol compared to the wild type). Gillman (2009) identified mutations in two soybean homologs of the maize *lpa1* gene giving evidence that two *lpa* homologs control the low phytic acid phenotype in soybean.

In maize two types of recessive mutants producing *lpa* phenotype are known, one is *lpa*-1, which has low phytic acid content and does not accumulate other inositol phosphates whereas the *lpa*-2 mutant does accumulate InsP3, InsP4 and InsP5 (Raboy et al., 2000). In 2003, the ZmIpk (Inositol Phosphate Kinase) gene was identified and it was reported this gene is involved in the biosynthesis of PA and *lpa*-2 mutants have a mutation in this gene. In 2005, the gene responsible for the *lpa*-3 was identified as a *myo*-ionsitol kinase (MIK) and is expressed in embryos, the phenotype exhibits low phytic acid (50 % less), increased *myo*-inositol and lacks lower phosphorylated intermediates in seeds (J. Shi et al., 2003, 2005).

Pilu (2003) proposed that the gene *MIPSIS* codes for the enzyme responsible of the first step in the biosynthesis of PA and it might cause the *lpa*241 phenotype, which has 90 % reduction on PA content and ten-fold increase in free phosphate in seeds. Unfortunately, this mutant displayed a number of pleiotropic effects such as low yield, slow growth and defective seedlings; embryos were smaller and contained fewer and smaller globoids. The fact that these effects were noticed in developmental stages other than seed maturation, suggested they had no relation

with the PA accumulation (Pilu et al., 2005). Studies in *Arabidopsis thaliana* proposed that the disruption of IPK1 and IPK2 could be a strategy for *lpa* crops. The mutants *Atipk1* and *Atipk2β* showed increased levels of bioavailable phosphorus by 10-fold and they do not accumulate phytate precursors; the yield was not affected but the plants were smaller, the authors suggested these could be overcome with breeding interventions (Stevenson-Paulik et al., 2005).

Later, Shi (2007), silenced a transporter in maize embryos and produced low phytic acid, high inorganic P seeds. The responsible *lpa-1* gene encoded a multidrug resistance-associated protein ATP-binding cassette transporter (MRP ABC transporter). It was named *MRP4* and is related to the *MRP5* of *Arabidopsis thaliana* and *OsMRP13* in rice. Two mutants lines produced, had PA reductions levels of 68-78 % and 32-75 % respectively. The strategy was tested in soybean producing seeds with 15-30-fold increase in Pi and 37- 90 % PA reduction. No significant adverse effects were observed in these plants making this gene a good candidate for developing *lpa* crops.

Dietary studies conducted with maize tortilla analysed the effect of a low-phytic acid maize on iron absorption in humans. Tortillas prepared form a wild type maize had 847 ± 20.35 mg 100 $g^{-1}$ (dry matter), whereas tortillas prepared from the *lpa* maize (*lpa1-1* mutant) had 368 ± 6.25 mg 100 $g^{-1}$ (dry matter). Mean molar ratios were: PA:Fe, 16.1 and 8.4, wild type and *lpa* respectively; PA:Zn, 32.9 and 14.0, wild type and *lpa* respectively. The iron absorption for *lpa* tortillas was 8.15 % meanwhile for the wild type strain was 5.48 %. Meaning that consuming the

average number of tortillas from *lpa* maize an iron-deficient woman would consume additional 0.45 mg Fe daily, which is about one third of the daily requirement. Although it was not tested directly in this study, because the PA:Zn ratio in the *lpa* strain was lower than the recommended threshold of 15, and the PAxCa:Zn was at the limit where zinc bioavailability is compromised (0.5), it is likely the consumption of tortillas made with the *lpa* strain are theoretically more favourable in terms of trace element absorption (Mendoza et al., 1998).

Similarly, another paper published the calcium intake from tortillas prepared from an *lpa-1-1* that had ~60 % PA reduction and a wild type as control. Calcium intake from both strains were 150 mg; PA concentrations in the *lpa* tortillas were 1.56 versus 3.0 mg $g^{-1}$ in the wild type, giving molar ratios Ca:PA of 10.9 and 4.9, respectively. Individuals absorbed significantly more Ca from the *lpa* maize tortillas ($0.50 \pm 0.03$) compared to the wild type ($0.35 \pm 0.07$). This meant 6 mg Ca extra would be absorbed per *lpa* maize tortilla (Hambidge et al., 2005).

In common bean (*Phaseolus vulgaris*), the *lpa* mutants presented 90 % reduction in the phytic acid content plus a 25 % reduction in raffinosaccharides. Moreover, the authors demonstrated that the mutant lines did not show the typical negative effects on agronomical performance (Campion et al., 2009). Other genes in the biosynthesis of PA in bean have been identified: *Myo*-inositol 1-phosphate synthase (*PvMIPSs and PvMIPSv*), Inositol monophosphatase (*PvIMP*), *Myo*-inositol kinase (*PvMIK*), Inositol 1,4,5-tris-phosphate kinase/inositol polyphosphate multikinase (*PvIPK2*), Inositol 1,3,4-triphosphate 5/6-kinase

(*PvITPKα, PvITPKβ*), Inositol 1,3,4,5,6 pentakisphosphate 2-kinase (*PvIPK1*) (Fileppi et al., 2010).

In barley, the *lpa1-1* phenotype was mapped and identified as a sulphate transporter (*HvST).* It was hypothesised this protein has a role in the transport of P to the PA synthesis pathway (Ye et al., 2011).

The literature referring to *lpa* wheat is more recent. The *lpa* line Js-12 LPA was the first low phytic acid to be reported in wheat. LPA wheat had three times more Pi than the WT in flour and 25 % more Mg than the WT in flour fractions, but the wholegrain P concentration was unaffected. Phosphorus and magnesium appeared to be redistributed in the kernel (Guttieri et al., 2006c). However, it was considered unacceptable from the agronomical perspective, the genotypes produced by this mutation presented reduced stature, weak straw and low grain yield (Guttieri et al., 2004). Later, agronomical studies were conducted to analyse populations derived from backcrossing Js-12 LPA in three different environments. The results suggested the environmental effect on PA and P concentrations might be just as great as the effect of genotype alone (Guttieri et al., 2006a).

Some genes in the biosynthetic pathway of phytic acid have been identified, for example, in *T. aestivum* L., the *MIPS* gene was characterised and the authors reported it is expressed in roots, stems, flag leaves and immature seeds at 15 days after flowering (DAF). It was also found that in cultivars with higher levels of PA the gene expression and enzymatic activity were higher compared with those with

lower PA content (D. Ma et al., 2013). Bhati (2014), identified for the first time some genes involved in the biosynthesis of inositol phosphates in wheat (*TaITPK1, TaITPK2, TaITPK3, TaITPK4, TaIPK2, TaIPK1*), and one gene homolog to the *Zmlpa-1* (*TaMRP3*). The authors suggested *TaIPK1* and *TaMRP3* are good targets for genetic engineering of low phytic acid wheat.

In 2016, the same authors reported their findings on the protein TaABCC13. The transgenic lines displayed a PA reduction of 34 and 22 % as well as an increase of Pi in mature grain. The viability on these plants were unaffected but other adverse effects in the spike development and delayed germination were present. Previous findings from Ali (2013) demonstrated that reduction of PA often causes a remobilization or change in micronutrient content. Bhati (2016) in his study with transgenic lines concluded this gene plays an important role in grain development and metal detoxification. It was observed the plants had an increase of Cu but Zn and Fe were not significantly affected, in addition, plants had defects on metal uptake and development of lateral root when exposed to cadmium stress.

A study that investigated the functional role of the pentakisphosphate kinase (TaIPK1) gene in wheat showed 25-56 % reduction of PA and increase of Pi of ~1.2 to 1.7-fold in the transgenic lines. The levels of iron were increased ~1.2 to 1.7-fold and a ~1.3 to 2.2-fold increase in zinc molar ratios. The seed count of the transgenic lines was affected, but no the seed weight or germination (Aggarwal et al., 2018).

LPA genotypes have demonstrated to cause changes in the distribution of minerals and in consequence the quality of these lines. Some investigations on the milling and baking quality of *lpa* genotype from Js-12-LPA in backcross-derived wheat lines showed an increase on the flour ash concentration but no detrimental effects on baking properties in hard wheat. Soft wheat on the other hand, displayed poor milling performance, possibly due to the damaged starch (Guttieri et al., 2006b; Jorge P. Venegas et al., 2018; Jorge Patricio Venegas, 2017).

Therefore having exposed this literature review, we present three experiments focused on the determination of phytic acid. In Chapter 2 a hydroponic system was used to analyse the concentration of PA in leaf and grain of wheat plants grown under different phosphorus and zinc supplies. We hypothesised that the plants accumulate more PA in grain at higher levels of P supplementation. Secondly, that it is possible to use leaf PA concentrations as proxy of the PA concentrations in grain. Third, we expect that some other mineral elements such as those highly linked with PA (Ca, Zn, Mg, Mn, Fe, Cu and K) are also affected by the treatments. In Chapter 3 and 4 we present the phytic acid variability of a diverse set genotypes and the contributions of the genotype, environment and G x E interaction. We also describe the PA ratios for all these genotypes, and report the potential bioavailability of Fe and Zn in these genotypes and highlight the importance of evaluating the phytic acid content.

# 2. EFFECT OF EXTERNAL PHOSPHORUS AND ZINC SUPPLY ON PHYTIC ACID CONCENTRATIONS ON WHEAT LEAF AND GRAIN

## 2.1 BRIEF INTRODUCTION

Paragon wheat plants were grown during three years in a nutrient film technique (NFT) hydroponic system. Phosphorus and zinc nutrient concentrations from a standard nutrient solution were modified to give four concentration levels. Phosphorus and zinc were tested independently. Samples of leaves during growth and grain at maturity were collected. Samples were analysed for mineral elements by ICP-MS and for phytic acid concentrations.

We hypothesised that the plants accumulate more PA in grain at higher levels of P supplementation. Secondly, we aim to explore the possibility of using the leaf mineral content as a proxy for their concentrations in grain. Third, we expect that manipulating the phosphorus and zinc nutrients will have an effect on the concentrations of other minerals such as those highly linked with PA (Ca, Zn, Mg, Mn, Fe, Cu and K).

## 2.2 AIMS AND OBJECTIVES

To generate knowledge on the relationship between the nutrient status of the plant with the rates of accumulation of phosphorus, phytic acid and other minerals in grain and leaves of *Triticum aestivum* L. A study was undertaken with the following objectives: 1) evaluate the effect of different levels of P and Zn on the accumulation of phytic acid (PA) and other minerals; 2) describe the relationships between PA and other minerals; and 3) assess the possibility to predict grain PA and mineral concentrations from their concentrations in leaves.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Plant material

Paragon wheat seeds coated with the fungicide Fludioxonil were used in these studies.

### 2.3.2 Plant growth and experimental design

Plant growth took place in glasshouse E in Sutton Bonington Campus of the University of Nottingham, with supplementary heating and lighting, to maintain conditions of at least 22 °C day 18 °C night, 12 h photoperiod. A summary of the experiments is presented in Table 2-1 below. For full details of plant identification numbers and corresponding treatments see Table 9-1.

Table 2-1 Summary of dates and names of the experiments performed.

| Set name | Treatment | Date |
|---|---|---|
| Set One | Phosphorus | 20 June 2016 to January 2017 |
| Set One | Zinc | 19 September 2017 to February 2018 |
| Set Two | Phosphorus and Zinc (independently) | 13 February 2018 to July 2018 |
| Set Three | Phosphorus and Zinc (independently) | 11 June 2018 to November 2018 |

The first phosphorus fertilization experiment and was conducted from the 20 June 2016 to January 2017. The following experiment tested zinc fertilization and was conducted from 19 September 2017 to February 2018, these two experiments were grouped in Set One (Table 2-1). The following two experiments tested both P and Zn, independently. They are referred in this document as Set Two, which was carried out from 13 February 2018 to July 2018 and Set Three, completed from 11 June 2018 to October 2018.

Detailed information about the experiments is provided in the following paragraphs.

### 2.3.3 Nutrient film technique (NFT) hydroponic system technical description

Hydroponics is a method of growing plants using mineral nutrient solutions in water without soil. There are many forms of hydroponics such as the nutrient film technique (NFT), drip system, aeroponics, Ebb Flow, water culture and wick system among others. The nutrient film technique is considered the most widely used system in hydroponics. The principle of this technique is that plants are grown with their roots contained in a plastic film trough or rigid channel through which nutrient solution is continuously, Figure 2-1. Allen Cooper at the Glasshouse Crops Research Institute in Littlehampton, England, pioneered the cropping technique in 1965. The term nutrient film technique was coined at the Glasshouse Crops Research Institute to stress that the depth of the liquid flowing past the roots of the plants should be very shallow in order to ensure that sufficient oxygen would be supplied to the plant roots (Mohammed, 2018a, 2018b; Resh, 2012).

The NFT hydroponic system used for our experiments consisted of a large metallic bench (503 cm x 95 cm x 92 cm) built with a 17 cm height difference from start to end. For the channels, four squared white gullies (400 cm x 12 cm x 5.5 cm; John A. Stephens Ltd.) fitted with two stop ends were placed on top.

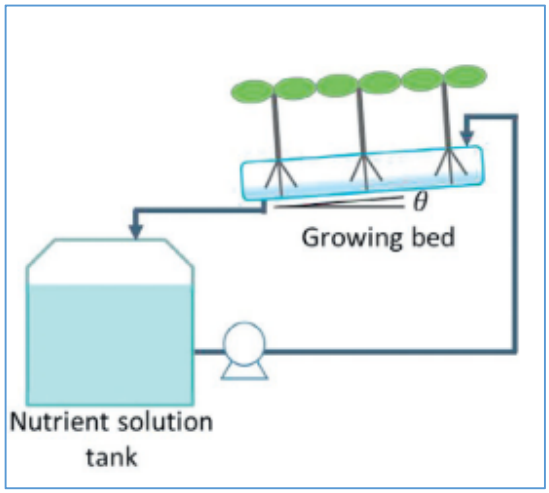



Figure 2-1 Hydroponic nutrient film technique (NFT) diagram. From Son (2015).

In the first phosphorus experiment, each of the gullies were covered with a plastic lid to which ten holes (32 mm diameter) were drilled to accommodate the plants. Plants did not have any extra support at this time.

For the second experiment (Set One Zinc) and onwards, the layout was slightly changed to accommodate rockwhool blocks (Grodan Delta Block, Delta 4.0 25/35; 75 mm x 75 mm x 65 mm). The lids covering the gully were drilled with squared holes (75 mm x 75 mm). On top of the blocks, an extra lid was laid to avoid algae growth, with a hole of 25 mm to sit the rockwool propagation plug. The separation between plants was approximately 22 to 22.5 cm, additionally, a wooden frame was fabricated to provide support to the plants (420 cm x 90 cm x 50 cm). See Figure 2-2 for photos. Figure 2-3 shows the layout of our hydroponic system. Fixtures and fitting for pipe work were supplied by Hortech Systems LTD: ¾ inch PE tank connector outlet bulkhead fitting, ¾ inch polypropylene back nut threaded

fitting, 20 mm nutlock elbow for LDPE pipe, 20 mm x ¾ inch nutlock valve for LDPE pipe, 16 mm x 20 mm nutlock connector joiner for LDPE pipe, 20 mm LDPE pipe. Under the tray four opaque black plastic boxes 84 L capacity were installed each of them adapted with a submergible pump running at the minimum flow rate (Aquarius Universal Classic 1500 L $h^{-1}$ max flow rate, Hortech Systems Ltd.).

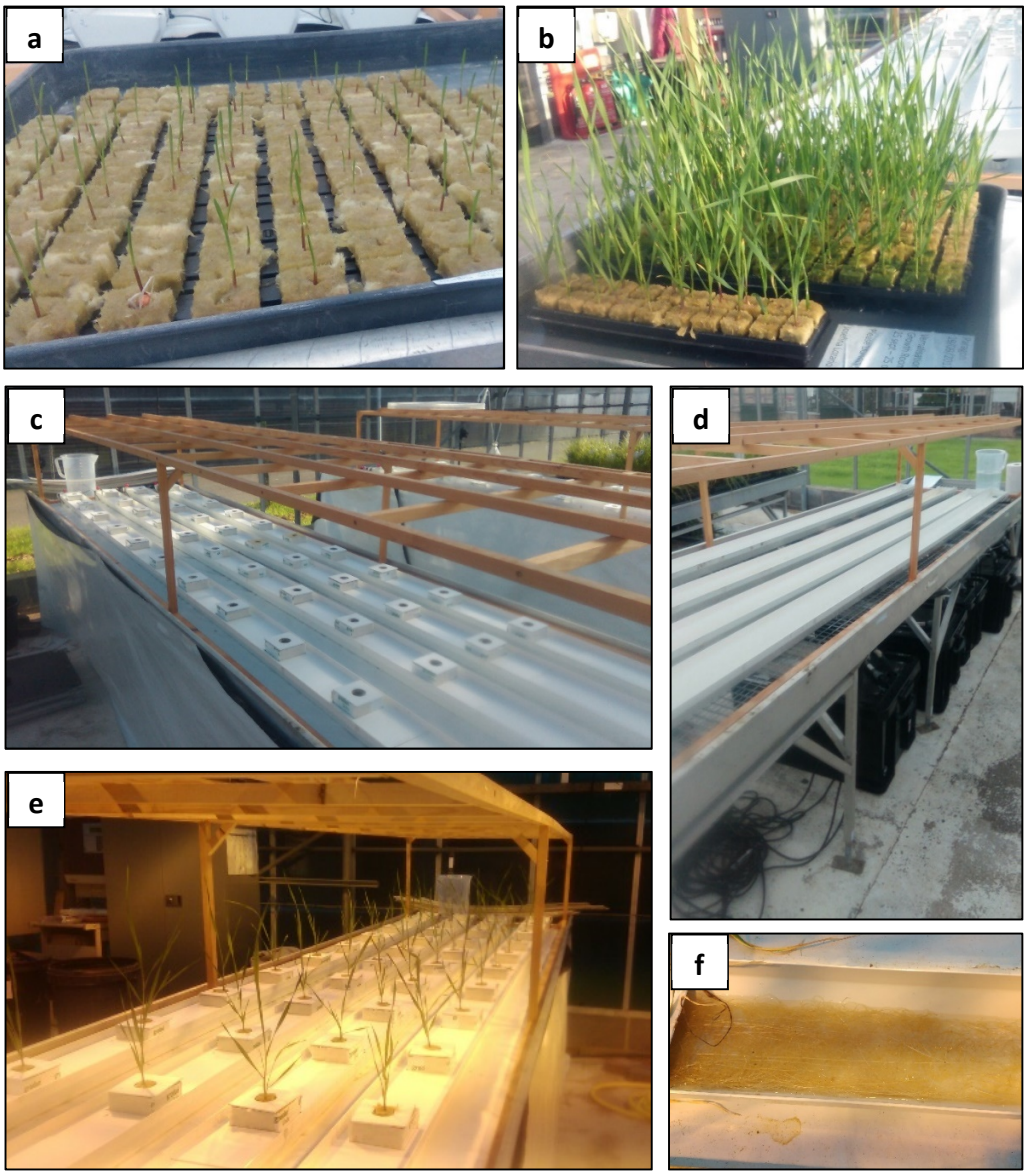



Figure 2-2 Paragon wheat plants in NFT system. a) Wheat seeds germinating in rockwool propagation plugs; b) Wheat seedlings after vernalisation period of four weeks; c) NFT hydroponic system set up with Grodan delta blocks and wooden frame support; d) NFT system showing containers for nutrient solution under the tray; e) Wheat seedlings freshly transplanted into NFT system; f) Wheat root growth of the last plant of each gully.

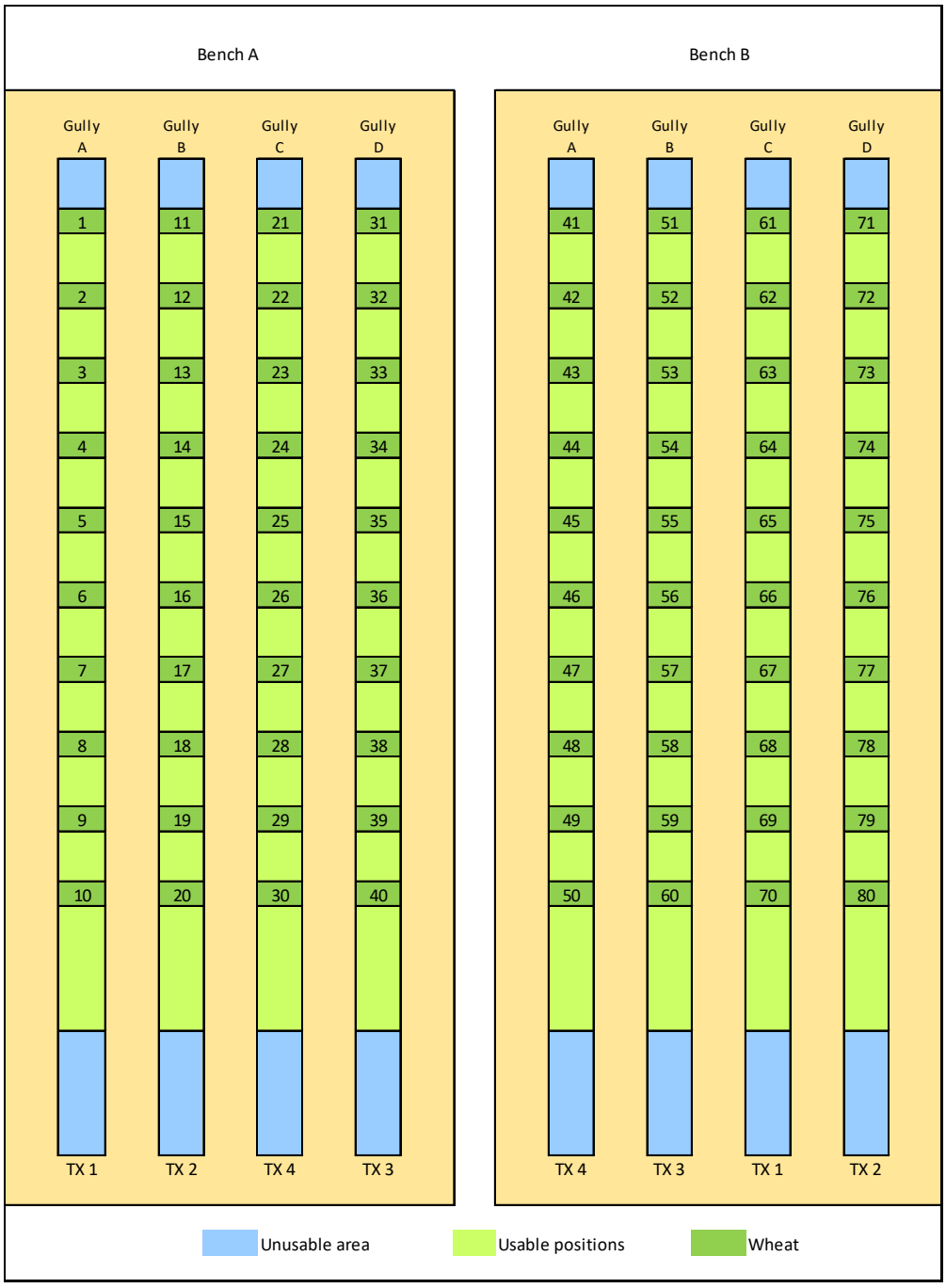



Figure 2-3 Nutrient Film Technique (NFT) hydroponic system arrangement of wheat plants.

### 2.3.4 Set one: Phosphorus june 2016 to january 2017

Seeds were sown in a 308-well propagation tray (609 mm x 400 mm x 45 mm, Desch Plantpak, Essex, UK) filled with Levington F2S compost (Scotts Professional, Ipswich, UK) and placed in the glasshouse to germinate. After seven days, the seedlings were put to vernalise in the cold growth room A09 located in the Phytotron building for four weeks. Seedlings were at growth stage 11 (GS 11) which according to the BBCH scale for cereals (Meier, 2001) corresponds to one leaf unfolded when moved to the cold room. The conditions in the cold room were 6 °C day, 8 °C night with a photoperiod of 12 hours. Plants were watered with reverse osmosis (RO) water (V2 Pure 50 Reverse Osmosis System). After the four weeks of vernalisation, plants were carefully removed from the germination tray leaving the compost to avoid root disturbance and transferred into a Nutrient Film Technique (NFT) hydroponic system built by Rory Hayden (technician). Seedlings were at GS 13 which corresponds to three leaves unfolded by the time they were transferred into the NFT system. See Figure 2-4 for plant photos.

The experimental design consisted of four different levels of phosphorus from a solution of $KH_2PO_4$ containing 1.9, 3.86, 7.72 and 15.45 mg $L^{-1}$ of phosphorus in the final solution with one level of zinc ($ZnSO_4$ .$7H_2O$ =1 µmol $L^{-1}$, equivalent to treatment 2 in the Zn experiment, Table 2-2. Each gully contained 10 plants. The whole system was replicated to have a total of 20 plants per treatment level. Which were labelled as bench A and bench B. Treatments were labelled as T1, T2, T3 and T4 respectively.

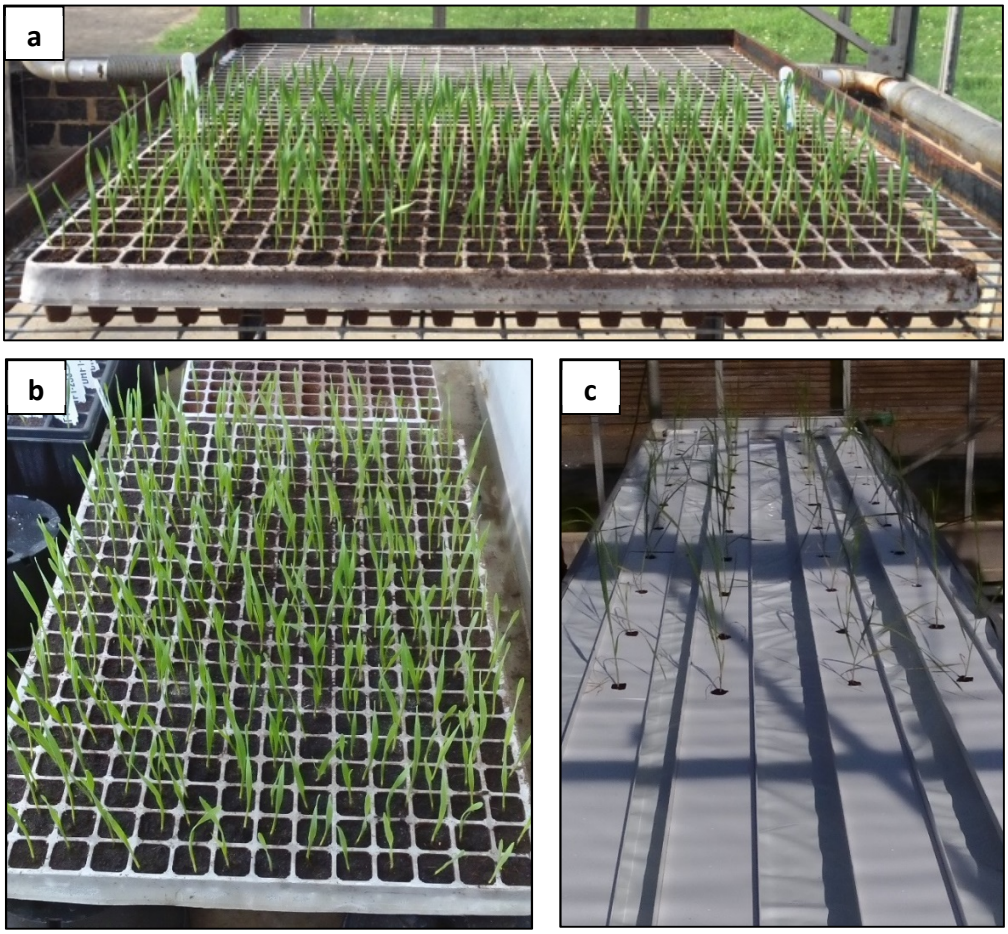



Figure 2-4 Paragon wheat seedlings. a) Seedlings after seven days in the glasshouse; b) Seedlings transferred to the cold room for vernalisation; c) Plantules transferred to hydroponic system in glasshouse after vernalisation period.

Treatments were randomly assigned to the gullies and the nutrient solution was freshly prepared and replaced once a week. To make sure both benches received the same concentration of nutrients; the total volume (60 L) for each treatment level was prepared in a single batch and then split into two. For key dates and detailed information of plant growth in the NFT system, see Table 9-2.

Table 2-2 Summary of treatments and their corresponding concentrations.

| **Nutrient** | **Level** | **$KH_2PO_4$ [final solution, mmol $L^{-1}$]** | **Phosphorus [final solution, mg $L^{-1}$]** |
|---|---|---|---|
| Phosphorus | T1 | 0.0625 | 1.9 |
| | T2 | 0.125 | 3.9 |
| | T3 | 0.25 | 7.7 |
| | T4 | 0.5 | 15.5 |
| | | $ZnSO_4.7H_2O$ [final solution, µmol $L^{-1}$] | Zinc [final solution, µg $L^{-1}$] |
| Zinc | T1 | 0.1 | 6.5 |
| | T2 | 1 | 65.4 |
| | T3 | 5 | 176.9 |
| | T4 | 10 | 653.8 |

### 2.3.5 Set one: zinc september 2017-february 2018

Seeds were sown in a 150-cell tray of Grodan 25 mm SBS (Single Block System) rockwool propagation cubes (Grodan (ROCKWOOL B.V.), KD Roermond, The Netherlands) and placed in the glasshouse to germinate and watered with RO water from the glasshouse system. After seven days, the seedlings were put to vernalise in the cold growth room A09 for four weeks. Growth room conditions were 6 °C day/night and a photoperiod of 12 hours. Once in vernalisation plants were fed with half-strength Hoagland's solution (Hoagland's No. 2 Basal Salt Mixture, Sigma-Aldrich Company Ltd.) prepared dissolving 0.8 g of powder in one litre of deionised water. Typically, one litre per week or as required. After the vernalisation period, plants were carefully removed from the propagation tray keeping the propagation plug and inserted in the rockwool blocks which were

placed in the hydroponic system. Seedlings were at GS 14 and some already had tillers (GS 21). See Figure 2-2 for photos.

The experimental design consisted of four levels of zinc and one level of phosphorus ($KH_2PO_4$ = 0.25 mmol $L^{-1}$, equivalent to treatment 3 in P experiment), Table 2-2. The setup of the NFT system was the same as the one used in the phosphorus experiment.

Zinc treatment levels were prepared from a solution of $ZnSO_4$ .$7H_2O$ containing 6.538, 65.38, 176.9 and 653.8 μg $L^{-1}$ of zinc in the final solution. Treatments were labelled as T1, T2, T3 and T4 respectively and were assigned to the gullies in the same order as in the phosphorus experiment to avoid confusion when preparing and changing solutions. The nutrient solution was freshly prepared and replaced once a week. For key dates and detailed information of plant growth in the NFT system see Table 9-2.

### 2.3.6 Sets two and three

Only the first attempt for each of the minerals was carried out in different years due to limited glasshouse infrastructure. The following experiments were carried out simultaneously, having a total of 160 plants per set. The plants form Set Two were sown in two batches, the second one of them did not germinate as expected and showed a different behaviour whilst in vernalisation, a possible explanation for this is that the seeds were left accidentally in the glasshouse and were exposed

to high temperature. For this reason, the plants were selected randomly and allocated in the NFT system randomly as well. For key dates and detailed information of plant growth in the NFT system Table 9-3. A summary of the treatments used and their concentrations can be found in Table 2-2.

### 2.3.7 Nutrient solution preparation

The nutrient solution was based on Hoagland's solution with some changes made to the original recipe.

For phosphorus experiment, the nutrient solution was prepared using seven stock solutions. Treatment levels were achieved by different proportions of two stock solutions of $KH_2PO_4$ and $K_2SO_4$ (solution 1 and 7 respectively). Solution number 7 is not originally included in the recipe but it was added to compensate the loss of potassium (from potassium sulphate $K_2SO_4$) in the lower treatments, and to achieve good pH buffering in the final solution.

The final nutrient composition was as follows: 0.0625, 0.125, 0.25 and 0.5 mmol $L^{-1}$ $KH_2PO_4$ (equivalent to 1.9, 3.86, 7.72 and 15.45 mg $L^{-1}$ of phosphorus), for treatment levels 1, 2, 3 and 4 respectively; 75 mmol $L^{-1}$ $MgSO_4.7H_2O$; 0.025 mmol $L^{-1}$ $CaCl_2.2H_2O$; 0.1 mmol $L^{-1}$ FeNaEDTA; 2 mmol $L^{-1}$ $Ca(NO_3)_2$; 2 mmol $L^{-1}$ $NH_4NO_3$; 0.23, 0.2 and 0.133 mmol $L^{-1}$ $K_2SO_4$ (for treatment levels 1, 2 and 3 respectively), and 0.93, 0.8, 0.53 mmol $L^{-1}$ KOH (for treatment levels 1, 2 and 3 respectively). For details of stock solutions, see Table 9-3, Table 9-4 and Table 9-5.

In terms of the micronutrients, a single stock solution was prepared and the concentrations were as follows: 30 µmol $L^{-1}$ $H_3BO_3$; 10 µmol $L^{-1}$ $MnSO_4.4H_2O$; 1 µmol $L^{-1}$ $ZnSO_4.7H_2O$; 3 µmol $L^{-1}$ $CuSO_4.5H_2O$ and 0.5 µmol $L^{-1}$ $Na_2MoO_4.2H_2O$. For details of stock solutions, see Table 9-4.

To prepare the solution each tank was filled up to 59.58 L with RO water. Then solution number 1, 2 and 7 were added, next the pH was adjusted to be just about 6 with 5 M $H_2SO_4$. Then the remaining solutions were added and the pH measured (pH Pal Plus pH tester; Electronic Temperature Instruments Ltd; Worthing, Sussex, United Kingdom) and adjusted to 6.3-6.4 with 5 M NaOH if necessary. The final 60 L solution was then split in half, so each of the benches would get 30 L solution. In warm days, the solution volume was increased to 40 L.

For zinc experiments nutrient solution was prepared using five macronutrient stock solutions and four micronutrient stock solutions. Zinc levels were achieved by varying the concentrations of $ZnSO_4.7H_2O$ in the micronutrient stock solution. Only one level of phosphorus was used in the zinc experiment (0.25 mmol $L^{-1}$ $KH_2PO_4$).

The final nutrient composition for macronutrients was as follows: 0.25 mmol $L^{-1}$ $KH_2PO_4$; 0.5 mmol $L^{-1}$ KOH; 75 mmol $L^{-1}$ $MgSO_4.7H_2O$; 0.025 mmol $L^{-1}$ $CaCl_2.2H_2O$; 0.1 mmol $L^{-1}$ FeNaEDTA; 2 mmol $L^{-1}$ $Ca(NO_3)_2$ and 2 mmol $L^{-1}$ $NH_4NO_3$.

Regarding the micronutrients, four stocks were prepared containing: 0.1, 1, 5 and 10 µmol $L^{-1}$ $ZnSO_4.7H_2O$ which is equivalent to 6.538, 65.38, 176.9 and 653.8 µg $L^{-}$

$^{1}$ of zinc in the final solution (treatments 1, 2, 3, 4 respectively) plus 30 µmol $L^{-1}$ $H_3BO_3$, 10 µmol $L^{-1}$ $MnSO_4.4H_2O$, 3 µmol $L^{-1}$ $CuSO_4.5H_2O$ and 0.5 µmol $L^{-1}$ $Na_2MoO_4.2H_2O$. For details of stock solutions, see Table 9-6, Table 9-7, Table 9-8 and Table 9-9.

To prepare the final solution, each tank was filled up to 59.58 L with RO water from the glasshouse system. Then solutions 1 and 2 were added, next the pH was adjusted to be just about 6 with 5 M $H_2SO_4$. Then the remaining solutions were added and the pH adjusted to 6.3-6.4 with 5 M NaOH if necessary. The final 60 L solution was then split in half so that each bench would get 30 L in total. In warm days, the solution volume was increased to 40 L.

When plants had flowered and most of the grain was filled (usually around day 113) the nitrogen supply was reduced by a quarter each week until the nitrogen source was reduced from 2 mmol L-1 to 0.5 mmol L-1 $NH_4NO_3$. To keep the plants from producing more tillers. However by mistake, in Set Two $Ca(NO_3)_2$ was reduced instead of $NH_4NO_3$, there were no noticeable consequences on plant growth and because the plants were in the last stages of maturation is very unlikely it could have affected grain composition.

### 2.3.8 Determining mineral concentrations in compost

Multielemental analysis and cation exchange capacity (CEC) assay was performed on F2S compost used to sow Paragon in the first P experiment. A representative

sample of compost was taken from the bag at the time of sowing and when the plants were transferred into the hydroponic system.

CEC procedure was done as follows: 2 g of air-dried compost samples were sieved to 2 mm. Extraction was done with 20 mL of 1 M $NH_4NO_3$ by shaking for 2 hours on a rotary shaker. Then the suspensions were centrifuged at 3500 rpm for 1 hour to ensure the top layer, which is lighter, went to the bottom. Next, the supernatant liquid was filtered through 0.22 µm polyether sulfone (PES) syringe filters. The filtered extract was diluted 1:10 in 2 % $HNO_3$. Three technical replications were then analysed using inductively coupled plasma-mass spectrometry (ICP-MS, Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific; Bremen, Germany) for Ca, Mg, K and Na as described by Thomas (2016).

Additionally, multielemental analysis was conducted on compost using the hydrofluoric acid (HF) digestion procedure for non-organic soil/compost as follows: 0.2 g air-dried compost samples were sieved to 2 mm. Extraction was done by incubating the samples overnight in 2 mL $HNO_3$ and 1 mL $HClO_4$ using a block digester for 8 h at 80 °C followed by another incubation of 2 h at 100 °C. The next day 2.5 mL of HF were added to the samples and incubated in a block digester as follows: 1 h at 120 °C, 3 h at 140 °C, 4 h at 160 °C. Once the temperature had dropped to 50 °C, 2.5 mL of ultrapure water (18.2 MΩ cm; Fisher Scientific UK Ltd, Loughborough, UK) were added and incubated for 1 hour at 50 °C. Finally, volume was made up to 50 mL with ultrapure water in plastic volumetric flasks. Three technical replicates were then analysed using ICP-MS to determine the

concentrations of 31 elements: Ag, Al, As, B, Ba, Be, Ca, Cd, Cr, Co, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, Tl, U, V and Zn. Blanks and the standard reference material (SRM) 2711a Montana II Soil (NIST; Gaithersburg, MD, USA) were also included.

The full procedure for HF digestion of soils includes a first step which is only used in organic soils, however as a recommendation from the laboratory technician this step was omitted. In detail, 4 mL of $HNO_3$ were added to the air-dried and sieved soil/compost sample, swirled and left for 30 minutes. After this the samples are incubated overnight using a block digester set with the following conditions, 50 °C 1 h, 80 °C 14 h, 30 °C hold. Next day the procedure continue where the non-organic HF digestion procedure starts.

Compost was digested using a Microwave Reaction System (Anton Paar GmbH; Graz, Austria), consisting of a Multiwave 3000 platform with a high-throughput rotor (48MF50) fitting 48 medium-pressure vessels (MF50) made of PFA, 50 mL volume supported by a fibre-reinforced PEEK vessel jacket.

The procedure was done as follows: 0.2 g of air-dried compost was digested in 2 mL concentrated Trace Analysis Grade (TAG) $HNO_3$, 1 mL ultrapure water (18.2 MΩ cm; Fisher Scientific UK Ltd, Loughborough, UK), and 1 mL $H_2O_2$. The settings were, 1400 W power, 140 °C and 2 MPa pressure for 45 minutes. After digestion, the tubes were made up to a final volume of 15 mL by adding 11 mL of ultrapure water, then transferred to 25 mL universal tubes (Sarstedt Ltd.; Nümbrecht,

Germany) and stored at room temperature. Prior to multielemental analysis by ICP-MS the samples were diluted 1:5 with ultrapure water in 13 mL ICP tubes (Sarstedt Ltd.). A duplicate of a certified reference material (CRM: wheat flour 1567b, NIST) and two operational blanks were included in each run.

The compost analysis were only performed in the first experiment (Phosphorus Set One) because the subsequent experiments were done in rockwool.

### 2.3.9 Determining mineral concentration in nutrient solution

Multielemental analysis of the nutrient solution was done by collecting samples of the freshly prepared solutions and from the residual solution in the boxes from the previous week in both benches (A and B). Samples were collected in 50 mL conical tubes, filtered using 0.22 µm syringe filters and kept at 4 °C. If the samples were to be kept for a longer time then they would be kept at -20 °C to prevent bacterial or algae growth and to eliminate any particle that may interfere with the correct functioning of the ICP-MS instrument.

The filtered solutions were acidified adding 400 µL of 50 % $HNO_3$ to 9.6 mL of sample in 13 mL ICP-MS tubes (Sarstedt Ltd.). Two technical replicates were analysed using ICP-MS to determine concentrations of 31 elements: Ag, Al, As, B, Ba, Be, Ca, Cd, Cr, Co, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, Tl, U, V and Zn. Each run included two replicates of reference materials, LRM

(Cabbage leaves) and CRM (wheat flour 1567b, NIST) and two operational blanks (RO water from the glasshouse).

### 2.3.10 Leaf and seed material sampling and preparation

Leaf and grain material were collected for multielemental analysis and phytic acid determination.

#### *2.3.10.1 Leaf material*

In Set One phosphorus, two leaves, the second and third leaf (top down) were collected from each plant tiller, see Figure 2-5. The first sampling of leaves was at the time of transplanting, the majority of plants were in GS 13. Subsequent collections were done at GS 47, which corresponds to flag leaf sheath opening or latter stages (when not possible). See Figure 2-6, Figure 2-7 and Figure 2-8 for details of the classification. Growth stage and date of all leaves collected were recorded. All samples were collected individually in paper bags and kept at -20 °C until freeze dried for three days at -83 C°. In order to have a representative sample for the analysis, all the leaves collected during the whole time course from the same plant were put together and manually crushed in the paper bag and then pulverised using a coffee grinder to obtain a homogeneous sample. For small samples, liquid nitrogen and a mortar and pestle were used instead to obtain a fine powder with a minimal loss. Powdered leaf samples were then used for phytic acid determination. For the microwave digestion and further ICP-MS analysis, leaf samples were crushed in the paper bags without using the coffee grinder.

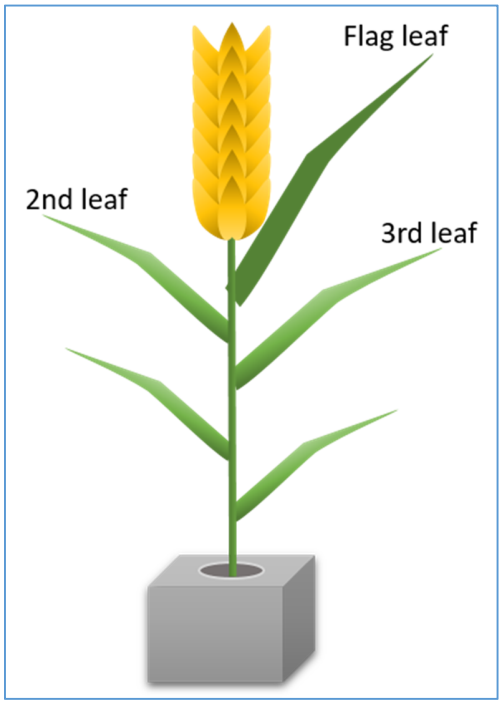



Figure 2-5 Diagram of a wheat plant showing the 2nd and 3rd leaves that were collected from each plant for further analysis.

In Set One Zinc, the sample collection and preparation changed a little. The same two leaves, the second and third leaf (top down) were collected, but this time all the plants were mature. Efforts were made to discard the senescent leaves and to remove leaf bits with large proportions of dead tissue (yellow colouring). All leaves from the same plant were put in the same paper bag and oven dried at 50 °C, instead of freeze drying them. Once dried they were crushed manually in the paper bag and pulverized using a hammer mill (Glen Creston Ltd; Stanmore, London). The result was a fine and very homogeneous powder which was stored for future use in 50 mL Falcon tubes with lids at room temperature.

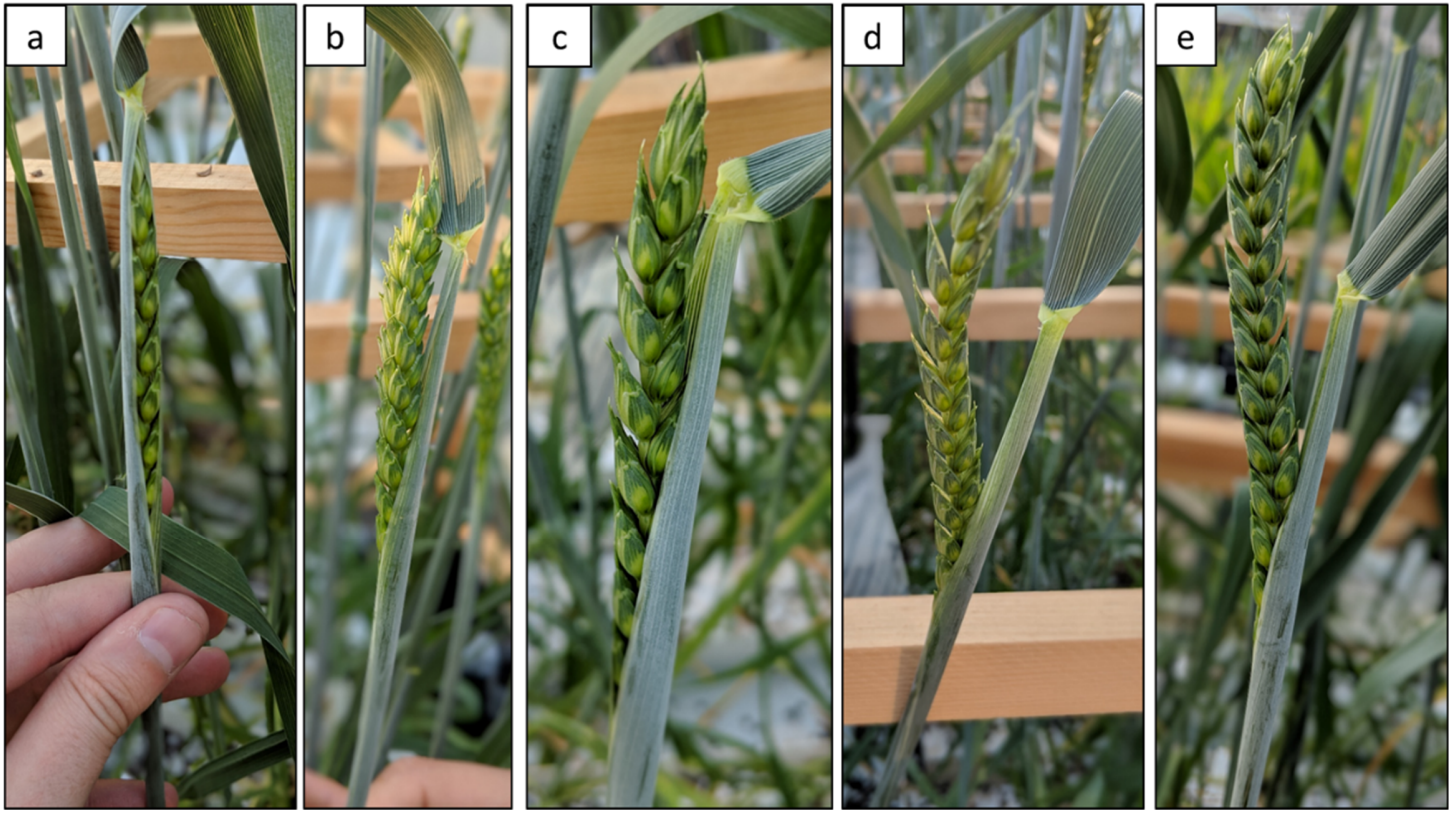



Figure 2-6 Paragon wheat in NFT hydroponic system. Photos show the growth stages (GS) used to classify plants for leaf sampling, taking as reference point the flag leaf. a) GS 47, flag leaf sheath opening; b) GS 51, beginning of heading: tip of inflorescence emerged from sheath, first spikelet just visible; c) GS 52, 20 % of inflorescence emerged; d) GS 53, 30 % of inflorescence emerged; e) GS 54, 40 % of inflorescence emerged.

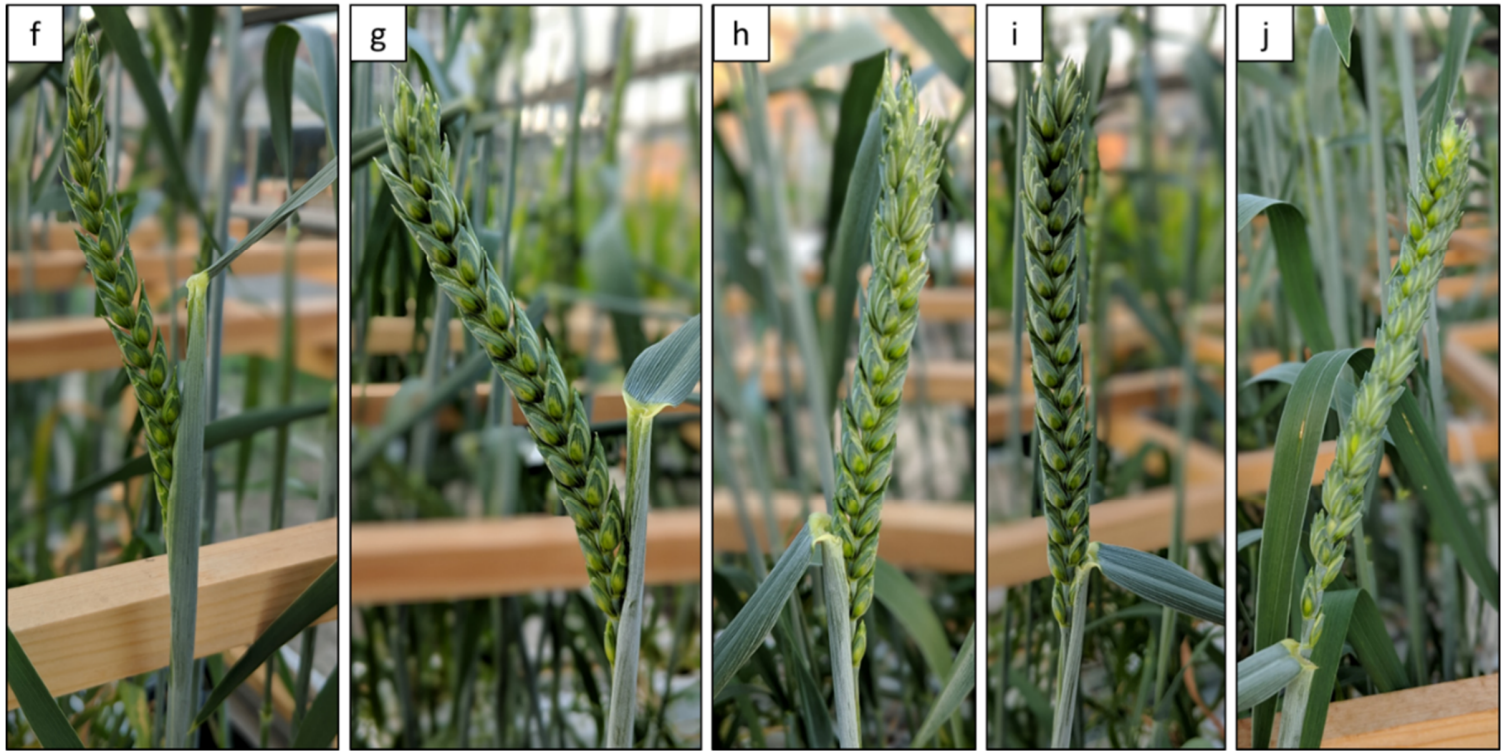



Figure 2-7 Paragon wheat in NFT hydroponic system. Photos show the growth stages (GS) used to classify plants for leaf sampling, taking as reference point the flag leaf. f-i) GS 55-58, 50-80 % of inflorescence emerged; j) GS 59, end of heading: inflorescence fully emerged.


Figure 2-8 Paragon wheat in NFT hydroponic system. Photos show the growth stages (GS) used to classify plants for leaf sampling. k) GS 61, beginning of flowering: first anthers visible; l-m) GS 62-63 some anthers are mature. Note that although not shown in here, GS 65 represents the full flowering stage with 50 % of the anthers mature.

For the following experiments (Set Two and Set Three). The second and third leaves (top down) were collected when the plants were in GS 47 avoiding fully flowered plants. Growth stage and date of collections were recorded. Leaves collected during the whole time course from the same plant were put together in a paper bag, oven dried at 50 °C and pulverized using the hammer mill mentioned before (Glen Creston Ltd.), and the resulting powder was stored for future use either in 50 mL plastic tubes or in brown envelopes in a really useful box at room temperature in the laboratory.

#### *2.3.10.2 Grain material*

Plants were harvested at the end of the cycle. Ears were counted, dehusked and hand cleaned. Grain was weighed and put in paper bags or brown envelopes for future use. Samples were pulverised with the hammer mill except the phosphorus-Set One where a coffee grinder was used instead.

In the Zinc Set One experiment, some plants had noticeable amounts of grain "aborted". The grain was sieved to 2 mm and when the defective grain accounted for more than 5 % of the total grain weight it was separated from the full grain and kept in a separate brown envelop. See Figure 2-9. Grain weight was expressed as grams per plant (g plant$^{-1}$).


Figure 2-9 Grain before (left tray) and after (right tray) sieving to remove incomplete seeds.

### 2.3.11 Determining mineral concentrations in leaf and grain samples

Leaf and grain samples from all three sets were digested using a microwave system and mineral concentrations were determined by ICP-MS. The procedures varied slightly with each experiment set, the following paragraphs describe the microwave acid digestion procedure.

#### *2.3.11.1 Pilot test for preliminary results*

The aim of this pilot test was to investigate if the treatments were working as expected. At day 108, one or two leaves (avoiding the flag leaf and regardless the leaf position) were sampled from the plants located at the beginning, middle and end part of the gully. Big leaves were selected in order to obtain at least 0.1 g of dry weight to perform the microwave acid digestion. Two replicates of laboratory reference material (LRM) cabbage and operational blanks were included. In total,

six plants form each treatment level were sampled. The protocol and instrument used for the digestion of these samples was the same as described previously in section 2.3.8.

#### *2.3.11.2 Set one microwave acid digestion*

The leaves and grain from two (beginning and end position) and three (beginning, middle and end position) plants of each gully, from phosphorus and zinc treatments respectively, were selected. Samples of 0.1 g and 0.2 g of leaves (phosphorus and zinc respectively) were digested in the microwave as previously described. For grain samples 0.2 g were used. Two replicates of LRM cabbage (0.2 g), CRM tomato leaves SRM 1573a, LRM Paragon wheat flour, CRM wheat flour SRM 1567b, and operational blanks were included in each run or accordingly depending on the sample tissue. See Table 2-3 and Table 2-4.

Table 2-3 Number of leaf samples used for microwave acid digestion and ICP-MS. Each sample is one plant grown in the NFT hydroponic system described earlier.

| Set | Treatment | Bench | T1 | T2 | T3 | T4 |
|---|---|---|---|---|---|---|
| 1 | Phosphorus | A | 2 | 2 | 2 | 2 |
| 1 | Phosphorus | B | 2 | 2 | 2 | 2 |
| 1 | Zinc | A | 3 | 3 | 3 | 3 |
| 1 | Zinc | B | 3 | 3 | 3 | 2 |
| 2 | Phosphorus | A | 3 | 3 | 3 | 3 |
| 2 | Phosphorus | B | 3 | 3 | 3 | 6 |
| 2 | Zinc | A | 3 | 3 | 3 | 2 |
| 2 | Zinc | B | 3 | 2 | 3 | 2 |
| 3 | Phosphorus | A | 3 | 3 | 3 | 3 |
| 3 | Phosphorus | B | 3 | 3 | 3 | 3 |
| 3 | Zinc | A | 3 | 3 | 3 | 3 |
| 3 | Zinc | B | 3 | 3 | 3 | 3 |

Table 2-4 Number of grain samples used for microwave acid digestion and ICP-MS. Each sample is one plant grown in the NFT hydroponic system described earlier.

| Set | Treatment | Bench | T1 | T2 | T3 | T4 |
|---|---|---|---|---|---|---|
| 1 | Phosphorus | A | 2 | 2 | 2 | 2 |
| 1 | Phosphorus | B | 2 | 2 | 2 | 2 |
| 1 | Zinc | A | 2 | 3 | 3 | 3 |
| 1 | Zinc | B | 3 | 3 | 3 | 3 |
| 2 | Phosphorus | A | 3 | 3 | 3 | 3 |
| 2 | Phosphorus | B | 3 | 3 | 3 | 6 |
| 2 | Zinc | A | 3 | 3 | 3 | 2 |
| 2 | Zinc | B | 3 | 2 | 3 | 2 |
| 3 | Phosphorus | A | 4 | 3 | 3 | 3 |
| 3 | Phosphorus | B | 3 | 3 | 3 | 3 |
| 3 | Zinc | A | 3 | 3 | 3 | 3 |
| 3 | Zinc | B | 3 | 3 | 3 | 3 |

#### *2.3.11.3 Set two microwave acid digestion*

In Set Two, for both treatments (P and Zn) the microwave digestion changed a little due to the acquisition of a new microwave system. The microwave system consisted of a Multiwave PRO platform with a 41HVT56 rotor fitting 41, 56 mL, high-performance pressure vessels with SMART-VENT Technology made of PTFE-TFM. The standard operating procedure (SOP) was done as follows: Approximately 0.2 g of leaf and wholegrain samples were digested in 6 mL of concentrated trace analysis grade $HNO_3$. Settings were: power 1500 W, 140 °C ramp for 20 minutes, 140 °C hold for 20 minutes and 55 °C cooling for 15 minutes.

After each run the microwave vessels were cleaned as indicated: rise vessels and caps with ultrapure water. Next add 3 mL ultrapure water and 3 mL concentrated $HNO_3$. Next, run the $HNO_3$ cleaning program (power 1500 W, 140 °C ramp for 10 minutes, 140 °C hold for 10 minutes and 55 °C cooling for 15 minutes).

After digestion, the tubes were made up to a final volume of 20 mL following the sequence: add 4 mL of ultrapure water into each digestion vessel, then transfer the sample to a clean 25 mL universal tube (Sarstedt Ltd., Nümbrecht, Germany), next add 5 mL to the digestion vessel and transfer it to the universal tube. Add a second aliquot of 5 mL to the digestion vessel and again transfer it to the sample in the universal tube.

Prior to analysis by ICP-MS the samples were diluted 1:10 with ultrapure water in 13 mL ICP tubes (Sarstedt Ltd.).

Two operational blanks were included in each digestion run as well as duplicates of certified reference materials (Wheat flour SRM 1567b, and tomato leaves SRM 1573a; NIST, Gaithersburg, MD, USA) and laboratory reference materials (LRM cabbage leaves and LRM Paragon wheat flour).

The leaves and grain from three plants of each gully (beginning, middle and end position), from phosphorus and zinc treatments, were selected, pulverised in the hammer mill and then used for the microwave digestion and analysed with ICP-MS as described above. See Table 2-3 and Table 2-4 for number of samples used.

#### *2.3.11.4 Set three microwave acid digestion*

In Set Three, the procedure was almost the same as the one just described for Set Two only with some changes to the microwave settings: The SOP was done as follows: Approximately 0.1 g of leaf and 0.2 g wholegrain samples were digested

in 6 mL of concentrated trace analysis grade $HNO_3$. Settings were: power 1500 W, 175 °C ramp for 20 minutes, 175 °C hold for 20 minutes and 55 °C cooling for 15 minutes. After each run the microwave vessels were cleaned as described for Set Two.

After digestion, the tubes were made up to a final volume of 24 mL and stored in universal tubes until ICP-MS analysis. Prior to analysis by ICP-MS the samples were diluted 1:10 with ultrapure water in 13 mL ICP tubes.

Two operational blanks were included in each digestion run as well as a duplicate of certified reference materials (Wheat flour SRM 1567b, and tomato leaves SRM 1573a; NIST, Gaithersburg, MD, USA) and laboratory reference materials (LRM cabbage leaves and LRM Paragon wheat flour).

The leaves and grain from three plants of each gully (beginning, middle and end position), from phosphorus and zinc treatments, were selected, pulverised in the hammer mill and then used for the microwave digestion and analysed with ICP-MS as described above. See Table 2-3 and Table 2-4 for number of samples used.

### 2.3.12 Phytic acid/total phosphorus quantification in Paragon wheat leaf and grain grown in NFT hydroponic system.

Leaves and wholegrain of selected plants grown in the NFT hydroponic system were processed to determine the phytic acid concentration using a commercial kit (phytic acid/total phosphorus; Megazyme International Ireland, Bray Co. Wicklow, Ireland), with slight modifications. The assay measures phytic acid as phosphorus

released by a phytase and an alkaline phosphatase. The principle of the method is presented in Figure 2-10 as described in the protocol supplied by the manufacturer.

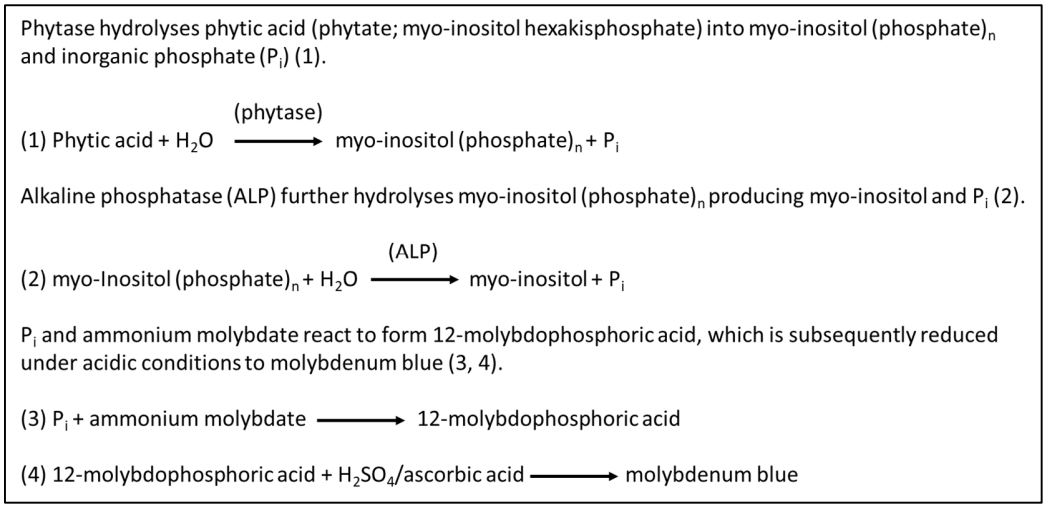



Figure 2-10 Assay procedure principle. From Megazyme (2014).

The procedure was performed in three main steps: sample extraction, enzymatic dephosphorylation reaction and colourimetric determination of phosphorus. These steps are detailed in the following paragraphs, firstly 1 g of the sample, was placed in a 50 mL conical tube to which 20 mL of 0.66 M HCl (Fisher Scientific UK Ltd.) were added. All samples had previously been milled and oven dried to make sure no extra moisture was present, samples were put in the oven at 50 °C at least 24 h before the analysis.

The extraction was then performed by shaking the tubes in a rotary shaker for at least three hours but for practicality, they were left overnight, for approximately 15 h. The following day, 1 mL of the acid extraction was taken and transferred into

a fresh 2 mL tube and centrifuged for 10 min at 13000 rpm. The supernatant was then neutralised by transferring it to a fresh 2 mL tube containing 0.5 mL of 0.75 M NaOH and vortexed. The neutralized extract was then ready to be used in the next step.

The second step was the enzymatic dephosphorylating reaction, where a series of buffers and enzymes included in the kit were added as per manufacturer's instructions. In order to do this, two sets of 2 mL tubes were labelled as free and total phosphorus. Then deionized water and buffer 1 (pH 5.5, plus 0.02 % w/v sodium azide as preservative) were added to both sets, followed by 50 µL of the sample extract, next the phytase suspension was added only to the set labelled as total phosphorus. Tubes were mixed by vortex and incubated in a water bath set at 40 °C for 10 min. Subsequently, deionized water and buffer 3 (pH 10.4, plus $MgCl_2$, $ZnSO_4$ and 0.02 % w/v sodium azide as preservative) were added to both sets plus the alkaline phosphatase suspension only to the total phosphorus set of tubes. Next, all the tubes were mixed by vortex and incubated in a water bath set at 40 °C for 15 min.

Next, the reaction was stopped by the addition of 300 µL of 50 % w/v trichloroacetic acid (VWR International, LLC.). The terminated reaction was then centrifuged at 13000 rpm for 10 min and 1 mL of the supernatant was carefully transferred to a fresh 2 mL tube for its use on the colourimetric determination.

For the colourimetric determination step, 0.5 mL of colour reagent (prepared by mixing 5 parts of solution A, ascorbic acid (10 % w/v)/sulphuric acid (1 M) with 1 part of solution B, ammonium molybdate (5 % w/v)), was added to the sample extract and the phosphorus standards calibration curve (described later), mixed by vortex and incubated in a water bath set at 40 °C for 1 hour.

After the incubation time the tubes were mixed by vortex and 300 µL were loaded into a 96-well plate and the absorbances read with the software Gen5 at 650 nm immediately after the process and never longer than three hours.

The calibration curve was prepared from a series of dilutions, from 0 µg to 7.5 µg (STD 0 – 4) of the phosphorus standard solution (50 µg $mL^{-1}$) provided in the kit. All samples were read in triplicates. On each run an operational blank (20 mL of 0.66 M HCl) was included.

For calculations, the raw data readings from free phosphorus, total phosphorus, control, LRM, blank and calibration curve were introduced in an excel spreadsheet (Microsoft Excel, 2016) and averaged.

Then the mean value of the blank was subtracted, using the corresponding blank for each set, by set meaning “free phosphorus sample” or “total phosphorus sample”. The resultant value was then introduced in the calculation program Mega-Calc™ that is provided via online by the company. The values of the calibration curve were averaged in excel but the blank was not substracted because this step is done automatically by the software (Mega-Calc™).

The concentration of phosphorus can be calculated as follows:

$$c = \frac{\text{mean M} \times 20 \times F}{10{,}000 \times 1.0 \times v} \times \Delta A_{phosphorus} \quad [g/100\ g]$$

**where:**

| | | |
|---|---|---|
| mean M | = | mean value of phosphorus standards [µg/$\Delta A_{phosphorus}$] |
| 20 | = | original sample extract volume [mL] |
| F | = | dilution factor |
| $\Delta A$ | = | absorbance change of sample |
| 10,000 | = | conversion from µg/g to g/100 g |
| 1.0 | = | weight of original sample material [g] |
| v | = | sample volume (used in the colourimetric determination step) |

Figure 2-11 Summary of the calculations steps. From Megazyme (2014).

The calculation procedure performed by the software provided by the manufacturer is as follows, first the absorbance of the STD 0 is substracted from the absorbance of the other standards (STD 1-4), thereby obtaining the absorbance difference (delta-Abs). From these values, a mean is calculated. The mean is then used to calculate the concentrations by multiplying it by the original sample extract (20 mL) and the dilution factor (55.6) and divided by 10000.

The result is then multiplied by the delta-Abs phosphorus, which is obtained subtracting the absorbance of the free phosphorus sample from the total phosphorus sample. The assay assumes that the phosphorus quantified is released entirely from phytic acid. Therefore, phytic acid is calculated by dividing the total

phosphorus concentration (g 100 $g^{-1}$) by 0.282, as 28.2 is the phosphorus ratio in the phytic acid molecule. Calculation steps are summarized in Figure 2-11.

### 2.3.13 Phytic acid to mineral molar ratio determination

Phytic acid to mineral millimolar ratios were obtained by first calculating the millimoles of PA dividing the concentration in mg $kg^{-1}$ by its molecular weight (MW) in g $mol^{-1}$ and equally with each of the mineral elements. Next, the resulting millimoles of PA are divided by the millimoles of the corresponding mineral. Molecular weight used were PA=660.04, Ca=40.08, Co=58.93, Cu=63.55, Fe=55.85, K=39.1, Mg=24.31, Mn=54.94, P=30.97 and Zn=65.4 g $mol^{-1}$.

### 2.3.14 Data cleaning and analysis

Data management and analysis were performed using GenStat 19th Edition (VSN International Ltd, Hemel Hempstead, UK), and Microsoft Excel 2016.

#### *2.3.14.1 Set one minerals*

In total, 16 leaf and grain samples in the phosphorus treatment were analysed with ICP-MS. For zinc treatments, 24 leaf and 23 grain (1 sample lost), were analysed. Consult Table 2-5 for number of samples per treatment, set and bench.

The following steps were used to clean the data from the ICP-MS multielemental analysis. First the LOD was calculated as three times the standard deviation of the operational blanks taking into account the sample's weight used for the analysis

(0.1 or 0.2 g); next the mean, standard deviation and limit of quantification (or upper limit) defined as the global mean plus five times the standard deviation, were calculated.

Those elements which did not have a good recovery (80-120 %) were excluded from further analysis. Then, mineral elements with a mean lower than the corresponding LOD were also eliminated from subsequent analysis. Next, individual values were inspected and those which were lower than the LOD were replaced with half the LOD value. This is called imputation and is done to deal with non-response bias, and therefore, the incomplete data set can still be analysed statistically. After this, individual values greater than the limit of quantification were also removed.

For leaf samples in phosphorus treatment, 11 mineral elements were removed from further analysis because the recovery was out of the reliable range (Ag, Al, Ba, Be, Cd, Co, Cr, Li, Ni, Se, U). The mean of some of these elements were also below the LOD value (Ag, Al, Be, Cd, Cr, Ni, Se, U).

For leaf of Zn treatments, the same elements were analysed and in this case the mean of six elements were below the LOD (Ag, Be, Cd, Cr, Ni, Se). Data were compared with the values obtained by Lolita Wilson for the LRM Cabbage because a CRM was not included for these samples, in this case, 11 elements were removed because the recovery was not reliable (Ag, Al, Ba, Be, Cd, Co, Cr, Li, Ni, Se, U).

For grain samples of P treatments, the values were compared with the CRM wheat flour and LRM cabbage if the value was not available for the CRM. In this case, the recovery of 12 elements was not reliable and were eliminated (Ag, As, Ba, Be, Cd, Co, Cr, Li, Na, Ni, Pb, U). Further five elements were removed because the mean was lower than the LOD (Al, Cs, Se, Sr, and V). Boron had 7 out of 16 values lower than the LOD, so it was also left out of further analysis.

For the grain samples of Zn experiment, 13 elements had a low recovery (As, Ba, Be, Co, Cr, Cu, Li, Ni, Pb, Se, Ti, Tl, U). The following elements had a mean lower than the LOD, the majority are included in the former (Ag, As, Be, Li, Pb, Se, Tl, U). Cadmium had 10 values out of 23 (43 %) that were lower than the LOD, and so was the case of Vanadium, therefore this were also eliminated from further analysis. From the elements with good recovery, individual data points lower than the corresponding LOD, were replaced with half the LOD value: one data point for B and seven for Al in the grain samples of Zn treatments. No values above the limit of quantification (global mean plus five standard deviations) were identified.

A summary of the elements that had a good recovery is on Table 2-6. For recovery data of all the elements, see Table 9-10.

Table 2-5 Detailed number of samples analysed with ICP-MS.

| | | Leaf samples | | Grain samples | |
|---|---|---|---|---|---|
| **Set** | **Treatment** | **A** | **B** | **A** | **B** |
| 1 | Phosphorus | 8 | 8 | 8 | 8 |
| 1 | Zinc | 12 | 11 | 11 | 12 |
| 2 | Phosphorus | 12 | 15 | 12 | 15 |
| 2 | Zinc | 11 | 10 | 11 | 10 |
| 3 | Phosphorus | 12 | 12 | 13 | 12 |
| 3 | Zinc | 12 | 12 | 12 | 12 |

Table 2-6 Reliable elements. Elements from which a good recovery was achieved (Recovery was between 80-120 %).

| Set | Treatment | Fraction | Element |
|---|---|---|---|
| 1 | Phosphorus | Leaf | B, As, Ca, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, P, Pb, Rb, S, Sr, Ti, Tl, V, Zn (n=20) |
| 1 | Phosphorus | Grain | Ca, Cu, Fe, K, Mg, Mn, Mo, P, Rb, S, Ti, Tl, Zn (n=13) |
| 1 | Zinc | Leaf | B, As, Ca, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, P, Pb, Rb, S, Sr, Ti, Tl, V, Zn (n=20) |
| 1 | Zinc | Grain | B, Al, Ca, Cs, Fe, K, Mg, Mn, Mo, Na, P, Rb, S, Sr, Zn (n=15) |
| 2 | Phosphorus & Zinc | Leaf | B, As, Ba, Ca, Co, Cu, K, Mg, Mn, Mo, Na, P, Rb, S, Sr, Zn (n=16) |
| 2 | Phosphorus & Zinc | Grain | B, Ba, Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, P, Rb, S, Sr, Ti, Zn (n=17) |
| 3 | Phosphorus & Zinc | Leaf | B, Ba, Ca, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Ni, P, Rb, S, Sr, Zn (n=17) |
| 3 | Phosphorus & Zinc | Grain | Ba, Ca, Co, Cr, Cu, Fe, K, Mg, Mn, P, Rb, S, Sr, Zn (n=14) |

#### *2.3.14.2 Set two minerals*

In total, 27 leaf and 21 grain samples from P and Zn treatments respectively. See Table 2-5 for number of samples per treatment, set and bench. Leaf and grain samples were collected described in section 2.3.10 (page 68). The procedure followed for the data tiding was the same as in Set One. For leaf samples (both P and Zn treatments), out of 31 elements measured, 12 elements were out of the reliable percentage of recovery and thus were eliminated (Ag, Al, Cr, Cs, Fe, Li, Ni, Pb, Se, Ti, U, V), additionally three elements were removed because the mean values across all treatments were lower than the LOD (Be, Cd, Tl). From the elements with good recovery, individual data points lower than the corresponding LOD, were replaced with half the LOD value: 13 data points for Na. No values above the limit of quantification (global mean plus five standard deviations) were identified.

For grain samples out of 31 elements measured, seven elements were out of the reliable percentage of recovery and thus were eliminated (Al, As, Cs, Na, Ni, Pb, V), additionally six elements were removed because the mean values across all treatments were lower than the LOD (Ag, Be, Cd, Li, Se, Tl, U). Selenium values were also removed because most of the values were negative. From the elements with good recovery, individual data points lower than the corresponding LOD, were replaced with half the LOD value: 12 data points for B. One data point for Ba, Co and Cr were above the LOQ. These individual values were removed.

A summary of the elements that had a good recovery is on Table 2-6. For recovery data of all the elements, see Table 9-11.

For the statistical analysis, only 10 elements were selected, based on the evidence that these are the most associated with phytic acid: Mg, P, S, K, Ca, Mn, Fe, Co, Cu, and Zn.

#### *2.3.14.3 Set three minerals*

In total, we analysed 24 leaf and 25 grain samples from P treatments and 25 leaf and 24 grain samples from Zn treatments, Table 2-5. The procedure to clean the data values of mineral elements was the same as the one described for Set One and Two.

For leaf samples, out of 31 elements measured, eight elements were out of the reliable percentage of recovery and thus were eliminated (Al, As, Li, Mo, Na, Pb, Ti, and V). Copper values were kept despite the recovery being 79.92 %. Additionally six elements were removed because the mean values across all treatments were lower than the LOD (Ag, Be, Cd, Se, Tl, U). No data points were substituted by half-LOD value. Three data points were removed from sample 325 (Cr, Co, Ni), because they were above the limit of quantification.

For grain samples out of 31 elements measured, five elements were out of the reliable percentage of recovery and thus were eliminated (Cs, Mo, Na, Ni, Ti). Additionally, 12 elements were removed because their mean values across all treatments were lower than the LOD value (Ag, Al, As, B, Be, Cd, Li, Pb, Se, Tl, U,

V). No data points were substituted by half-LOD value. No values above the limit of quantification were identified.

A summary of the elements that had a good recovery is on Table 2-6. For recovery data of all the elements, see Table 9-12.

For statistical analysis, only nine elements were selected from each of the sets, based on the evidence that these are the most associated with phytic acid: Ca, Co, Cu, K, Fe, Mg, Mn, P, and Zn. See Table 9-13 for number of values by set, treatment and bench.

#### *2.3.14.4 Phytic acid data*

A total of 289 samples plus controls and blanks were analysed for phytic acid concentration of which 148 were leaf (76 from P treatments and 72 from Zn treatments) and 141 (72 from P treatments and 69 from Zn treatments) grain samples. Detailed number of values per set, treatment and bench are Table 2-7.

Table 2-7 Number of samples analysed for phytic acid.

| | | Leaf | | Grain | |
|---|---|---|---|---|---|
| **Set** | **Treatment** | **A** | **B** | **A** | **B** |
| 1 | Phosphorus | 12 | 13 | 9 | 11 |
| 1 | Zinc | 14 | 13 | 12 | 12 |
| 2 | Phosphorus | 12 | 15 | 12 | 15 |
| 2 | Zinc | 11 | 10 | 11 | 10 |
| 3 | Phosphorus | 12 | 12 | 13 | 12 |
| 3 | Zinc | 12 | 12 | 12 | 12 |

Effort was made to match the grain and leaf samples from the same plant but when not possible, another plant with more available plant material was included in order to reach a minimum of three replicates per treatment level. We had only two cases in this circumstances.

The kit's manufacturer states that the smallest differentiating absorbance for the assay is 0.005 absorbance units which corresponds to a phosphorus concentration of ~ 28.2 mg $kg^{-1}$ of original sample (or phytic acid concentration of ~ 1000 mg $kg^{-1}$); and that under the conditions of the standard assay procedure the LOD is ~ 112.9 mg $kg^{-1}$ phosphorus of original sample (or ~ 400 mg $kg^{-1}$ phytic acid), which is derived from an absorbance difference of 0.020. However, because we used a 96-well plate instead of individual one centimetre light path cuvettes; and the wavelength at 650 nm instead of 655 nm. We decided to recalculate the LOD using our own blank values.

Firstly the delta-Abs (difference in absorbance) values of each blank were averaged and the LOD was reported as three times the standard deviation (SD).

To obtain the corresponding concentration of phosphorus and hence phytic acid, the LOD value was introduced in the Mega-Calc™ software assuming 1 g of weight of original sample, 20 mL of extraction solution and using the corresponding averaged calibration curve.

Then, for any individual sample that had a delta-absorbance below the LOD value, it was replaced with 50 % the LOD and thus its corresponding phosphorus and phytic acid concentration changed.

After substituting any values lower than the LOD, the mean and SD were calculated separately for leaf and grain samples. Next, to identify any outliers, we calculated a limit of quantification (LOQ) which we defined as the global mean plus five times the standard deviation. No data points were higher than the LOQ therefore, no data points were excluded in either set of samples.

#### *2.3.14.5 Dilution effect*

To take into account any possible dilution effect, the concentrations of phytic acid were re-calculated using the grain yield. The phytic acid concentration expressed in mg $kg^{-1}$ were multiplied by the grain yield values (g per plant) and the result was divided by 1000 to obtain PA concentrations in mg per plant.

## 2.4 RESULTS

### 2.4.1 Pilot test and preliminary results

Out of the 31 mineral elements, five (Ag, Be, Cr, Tl, U) were removed and left out of the analysis because the mean concentration was lower than the limit of detection (LOD). Then, individual values that were lower than the LOD were substituted with half the LOD value. Recovering values were calculated comparing the values obtained from the CRMs or LRMs. When this was not possible (CRM or LRM not included in the digestion run, CRM not providing the value for a specific element, etc.), the values were compared with those obtained by Lolita Wilson (lab technician).

Recovery can vary depending on multiple factors, for example: the biological sample does not have that particular element or its concentrations are very low, the preparation of reagents, contaminating particles in samples or standards for calibration, interferences, error derived from the measuring instrument, etc. In our samples, recovery was acceptable when the value was between 80-120 % of the true value, which is the standard rule for accuracy (± 20 %) in our lab. For this reason further 10 elements (Al, B, Ba, Cd, Co, Li, Ni, Pb, Se, and V) were eliminated.

In the pilot test, phosphorus mean concentration values for leaf samples were 9399, 13072, 12899 and 13285 mg $kg^{-1}$ for P treatment 1, 2, 3 and 4 respectively ($n = 6$). The analysis of variance showed a significant difference at the 5 % ($p = 0.018$) for treatment 1 (LSD = 2652.5). Blocking by bench did not make any further

difference. We decided to continue with the same treatments after we saw they were having an effect.

### 2.4.2 NFT leaf phosphorus experiment

In total, 76 samples of leaf were analysed in three sets. The phytic acid concentration ranged between 136.5 mg $kg^{-1}$ to 5348 mg $kg^{-1}$. The real minimum was 376.72 mg $kg^{-1}$, but the values substituted for half-LODs (11 data points) were also included in this range. The phytic acid median for each of the three sets was, 899.09, 976.18 and 1212.87 mg $kg^{-1}$ for Set One, Two and Three respectively.

In Set Two, eleven values were substituted for half-LOD values, these corresponded to four data points from treatment 1 and 2, one data point from treatment 3 and two data points from treatment 4. In Set Three, there were no values lower than the calculated LOD. Limit of quantification (LOQ) was calculated as the global mean plus five times the standard deviation. This was done per set individually. No values were higher than the calculated LOQ, for calculation details see Table 9-14.

For leaf samples the mean values of phytic acid for Set One, Two and Three were, respectively: 1265.83 ± 1313, 1362.67 ± 1028, 1456.36 ± 919 mg $kg^{-1}$ (mean ± SD).

Unbalanced ANOVA analysis showed no significant differences between the sets ($p=0.709$). In addition, there were not differences between the benches ($p=0.718$),

therefore the analysis was not blocked. Table 2-8 shows a summary of phytic acid mean, median and SD values by set and bench.

Table 2-8 Phytic acid mean, median and SD by set and bench of leaf samples in an NFT Phosphorus experiment. Data are in mg $kg^{-1}$.

| | | Bench A | | | | Bench B | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Set** | **P treatment level** | **n** | **Mean** | **SD** | **Median** | **n** | **Mean** | **SD** | **Median** |
| 1 | 1 | 3 | 676 | 437 | 916 | 4 | 379 | 240 | 376 |
| 1 | 2 | 3 | 362 | 165 | 454 | 3 | 172 | 0 | 172 |
| 1 | 3 | 3 | 2070 | 807 | 1917 | 3 | 1588 | 939 | 1208 |
| 1 | 4 | 3 | 2490 | 2630 | 1949 | 3 | 2685 | 649 | 2787 |
| 2 | 1 | 3 | 442 | 333 | 393 | 3 | 668 | 82 | 713 |
| 2 | 2 | 3 | 808 | 163 | 795 | 3 | 839 | 108 | 839 |
| 2 | 3 | 3 | 1089 | 825 | 1547 | 3 | 2549 | 2012 | 1552 |
| 2 | 4 | 3 | 2095 | 268 | 2017 | 6 | 1887 | 905 | 2179 |
| 3 | 1 | 3 | 448 | 118 | 384 | 3 | 503 | 112 | 533 |
| 3 | 2 | 3 | 1200 | 75 | 1191 | 3 | 1036 | 186 | 1005 |
| 3 | 3 | 3 | 1267 | 570 | 1257 | 3 | 1937 | 951 | 2035 |
| 3 | 4 | 3 | 2952 | 239 | 2896 | 3 | 2307 | 509 | 2592 |

SD, standard deviation; NFT, Nutrient Film Technique; P, phosphorus.

Normality was evaluated using skewness and kurtosis data, Shapiro-Wilk test and Q-Q plots with 95 % confidence limits. In this case for example, Shapiro-Wilk test for normality was significant ($p<0.001$). Because transforming data is not recommended, the analysis was performed first assuming normality with an unbalanced ANOVA. Further on this section, data is analysed removing “outliers” and also with a non-parametric test. The corresponding results are shown.

Assuming normality of the data we first tried an unbalanced ANOVA. The results showed that the treatments had a highly significant effect on the phytic acid concentration in leaf samples ($p<0.001$), whereas the set and the bench were not significantly different. Fisher's least significant difference (LSD) test for multiple comparisons generated two clusters and showed that treatments 1 and 2 are significantly different from treatments 3 and 4. Mean concentrations for each treatment were 512, 736, 1750 and 2329 mg $kg^{-1}$ for T1-4 respectively. Treatments are labelled as: treatment 1 (0.0625 mmol $L^{-1}$ $KH_2PO_4$), treatment 2 (0.125 mmol $L^{-1}$ $KH_2PO_4$), treatment 3 (0.25 mmol $L^{-1}$ $KH_2PO_4$) and treatment 4 (0.5 mmol $L^{-1}$ $KH_2PO_4$). Figure 2-12, top chart.

The percentage of Phytic acid-P/total P was 8.7 % for treatments 1 and 2, 11.7 % for treatment 3 and 7.7 % for treatment 4, there was no significant difference between the treatments ($p=0.163$). To perform this calculation, phytic acid-P, determined by the Megazyme Kit, is compared to the total phosphorus, which is obtained by ICP-MS analysis, and then expressed as a percentage. Figure 2-12, bottom chart.

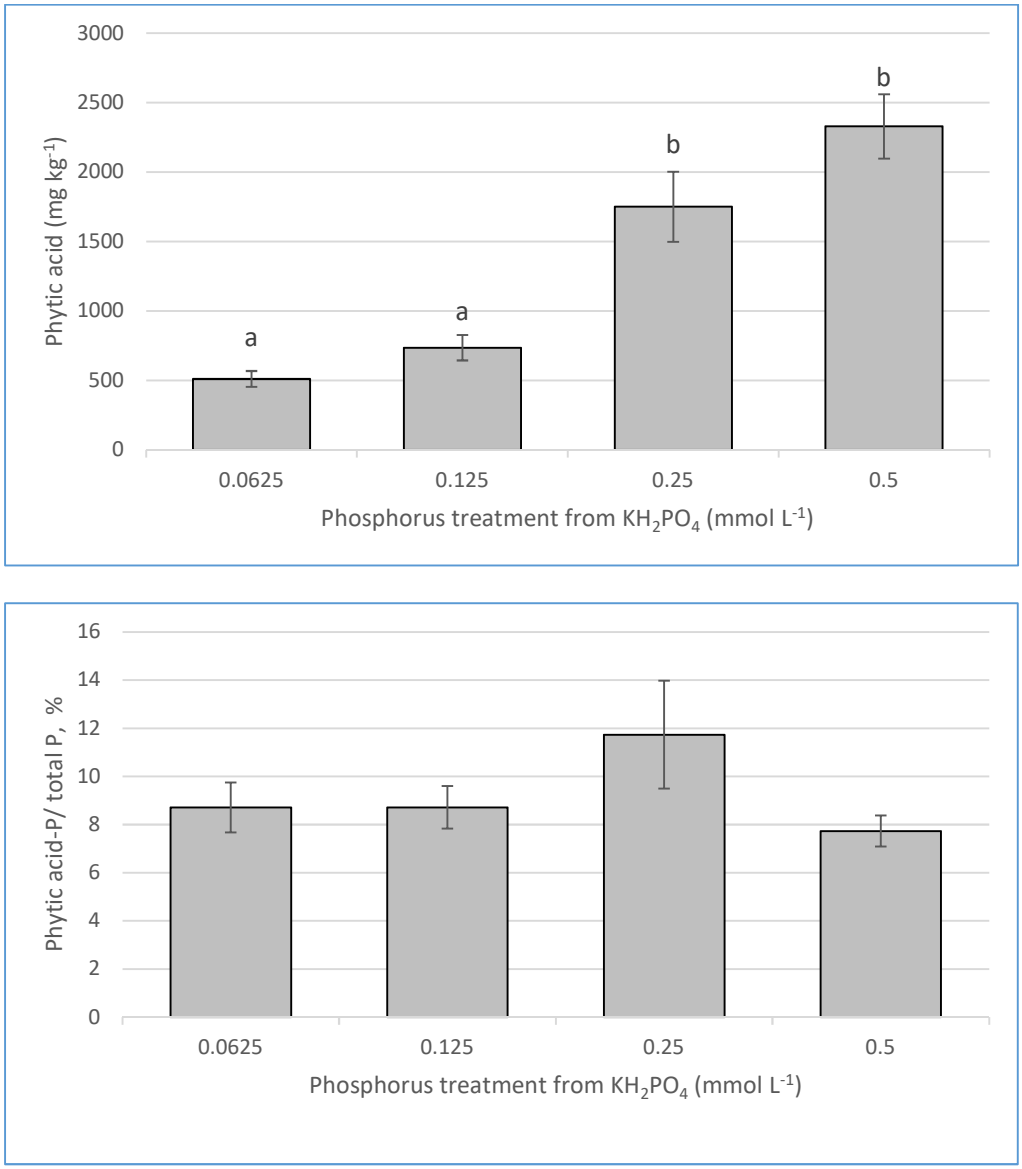



Figure 2-12 Phytic acid concentration in leaf samples by treatment applied in the hydroponic system. Three-year data. Top chart, mean values ± standard error bars (SE). Means followed by a common letter are not significantly different by Fisher's unprotected LDS test at the p=0.01 level of significance. Bottom chart, Phytic acid-P/total P %, mean ± SE bars. T1 n=19, T2 n=18, T3 n=18, T4 n=21. Phosphorus treatment concentrations are in millimole per litre (mmol $L^{-1}$). T1=0.0625 mmol $L^{-1}$, T2=0.125 mmol $L^{-1}$, T3=0.25 mmol $L^{-1}$, T4=0.5 mmol $L^{-1}$ $KH_2PO_4$.

#### *2.4.2.1 Trying statistical tests and removing "outliers" to normalise data*

To test for normality, several tests were employed, but this time, it was tested treatment by treatment, in this case, treatment 1 and 2, were normal according to Shapiro-Wilk's test, Q-Q normal plots and it was also verified that the skewness and kurtosis were no greater than twice their corresponding standard error. In case of treatment 3, all tests failed. However if the highest point of this treatment (4865.07) is eliminated, then the data is normalised. However this point is not greater than the established upper limit or limit of quantification, defined at the moment of cleaning the data, as the global mean plus five times the standard deviation. Treatment 4 had the same problem, in this case the kurtosis was greater than twice the standard error of kurtosis, and regarding the data point that seems to be an outlier (5347.97), kurtosis is fixed when this point is set as missing but now the skewness is jumbled, the problem were the two lowest data points that represent half the LOD substitutions (imputations). If we eliminate these four "outliers", the data becomes normal and a further unbalanced ANOVA shows that the treatments are significantly different ($p<0.001$), average LSD=164.8 (Fisher's unprotected LSD test, 1%) the means changed a little: 512, 736, 1567 and 2403 mg $kg^{-1}$ for treatments 1-4 respectively, Figure 2-13.

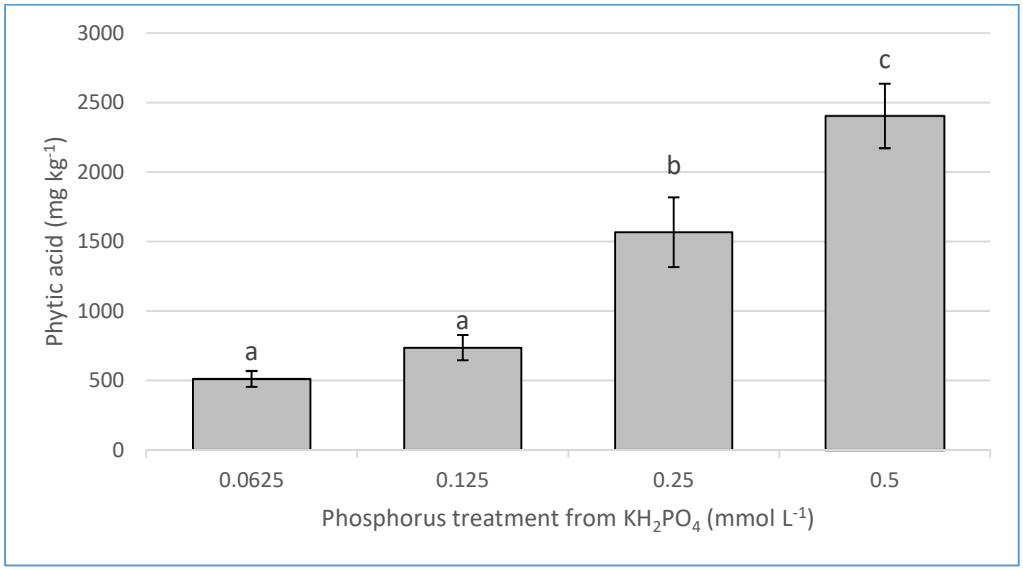



Figure 2-13 Phytic acid concentrations in leaf samples as affected by the phosphorus supply in a hydroponic system with "outliers" eliminated. Three-year data. Means followed by a common letter are not significantly different by Fisher's unprotected LDS test at the p=0.01 level of significance. T1, 0.0625 mmol L-1 n=19; T2, 0.125 mmol L-1 n=18; T3, 0.25 mmol L-1 n=17; T4, 0.5 mmol L-1 n=18.

Phytic acid-P/total P % remained almost the same and with no significant differences between the treatments, mean values were 8.7, 8.7, 9.9 and 7.7 % in treatments 1-4 respectively.

With "outliers" included, the correct analysis for non-normal data is a non-parametric analysis, to illustrate this, we ran a Kruskal-Wallis one-way ANOVA of one variate with group factor. Results showed a Chi-square probability of <0.001, see Table 2-9 for results.

To analyse differences between treatments, we used a Mann-Whitney U test setting treatment 3 as the control group (because this treatment has the standard

concentration of phosphorus in the hydroponic solution used in our lab). We tested the probability of null hypothesis that each group is equal to the control group. The result can be found in the last column (Mann-Whitney U test probability) in Table 2-9. We observe that treatment 1 and 2 had PA concentrations significantly lower than treatment 3 ($p<0.001$), and on the contrary, treatment 4 had higher PA concentration compared to the control group ($p=0.022$).

Table 2-9 Phytic acid concentrations of wheat leaves grown in a hydroponic system with four phosphorus treatments. Analysis by non-parametric test Kruskal-Wallis one-way ANOVA and Mann-Whitney U test.

| **Treatments** | **Size** | **Mean rank** | **Mann-Whitney U test (Chi-square probability)** |
|---|---|---|---|
| Treatment 1 | 19 | 18.66 | 26.5 (<0.001) |
| Treatment 2 | 18 | 27.33 | 48.0 (<0.001) |
| Treatment 3 | 18 | 48.39 | Control |
| Treatment 4 | 21 | 57.55 | 108.5 (0.022) |
| Value of H = 39.17<br>Adjusted for ties = 39.22<br>Degrees of freedom = 3<br>Chi-square probability <0.001 | | | |

Kruskal-Wallis one-way ANOVA and Mann-Whitney U test were performed in a group independent way.

### *2.4.2.2 Phytic acid and minerals in leaf samples*

To evaluate if there was relationship between the Phytic acid-P/total P % and total P in leaves, a correlation analysis was performed. There was no significant relationship between the two variables in leaves, ($r= -0.157$, $p=0.204$), Figure 2-14.

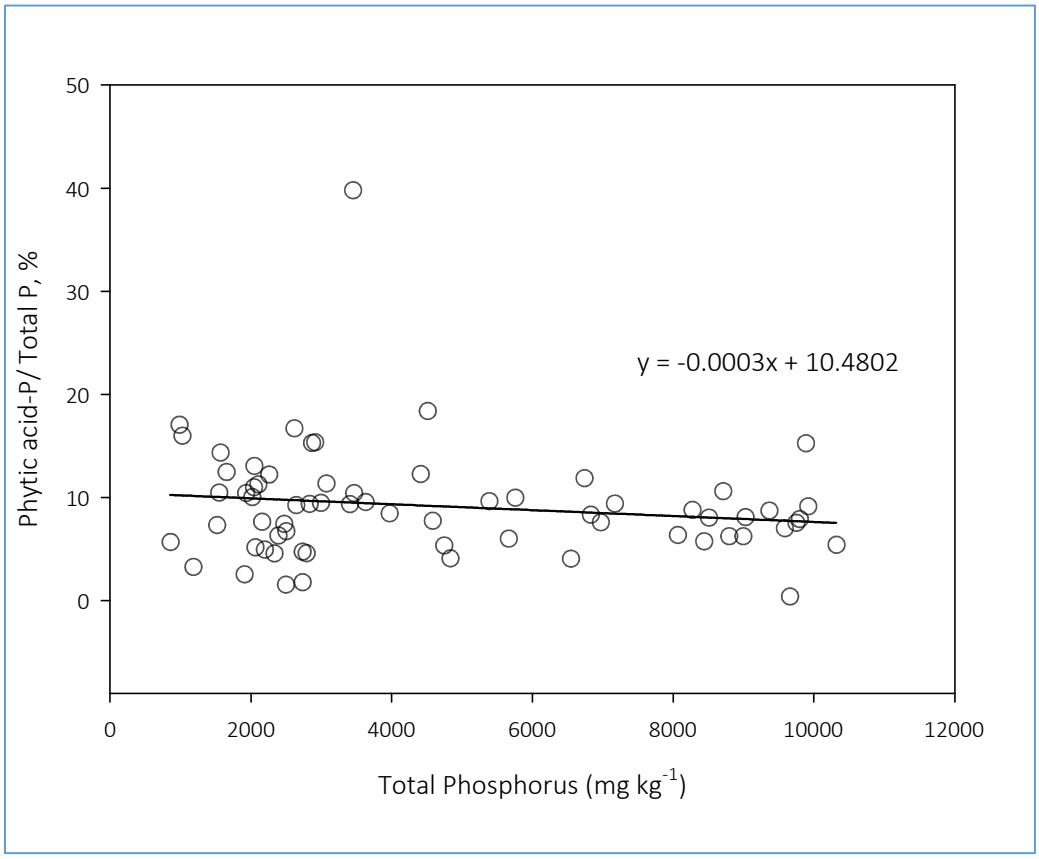



Figure 2-14 Relationship of Phytic acid-P/total P % and total P (mg $kg^{-1}$) in wheat leaves grown in a hydroponic system under four different P treatments, n=67.

Leaf phytic acid concentration showed a strong positive significant relationship with leaf Ca (r=0.634, p=0.0012), Mg (r=0.660, p<0.001), Mn (r=0.832, p<0.001), P (r=0.897, p<0.001) and Zn (r=0.770, p<0.001). Phosphorus was significantly associated to Ca (r=0.830, p<0.001), Mg (r=0.833, p<0.001), Mn (r=0.960, p<0.001) and Zn (r=0.890, p<0.001) concentrations, Figure 2-15.

These minerals were analysed by a simple linear regression with groups and set as parallel lines, to see if the phytic acid accumulation was affecting mineral

concentrations in leaf, phytic acid was set as the independent variable. Some interesting results were found, for example significant linear models ($p<0.001$) were found for Ca, explaining 75.3 % of the variation, K (20 %), Mg (78 %), Mn (64.7 %) and P (89.7 %). Observing the heat map (Figure 2-15) it is evident that calcium shows strong associations with Mg, Mn, P and Zinc, therefore a linear model could have been explored, however these are complex interactions and possibly go further than a linear regression, requiring a deeper understanding and knowledge and more advanced expertise in mathematical modelling, plus it might be out of the scope of the present study. Therefore these will not be further discussed.

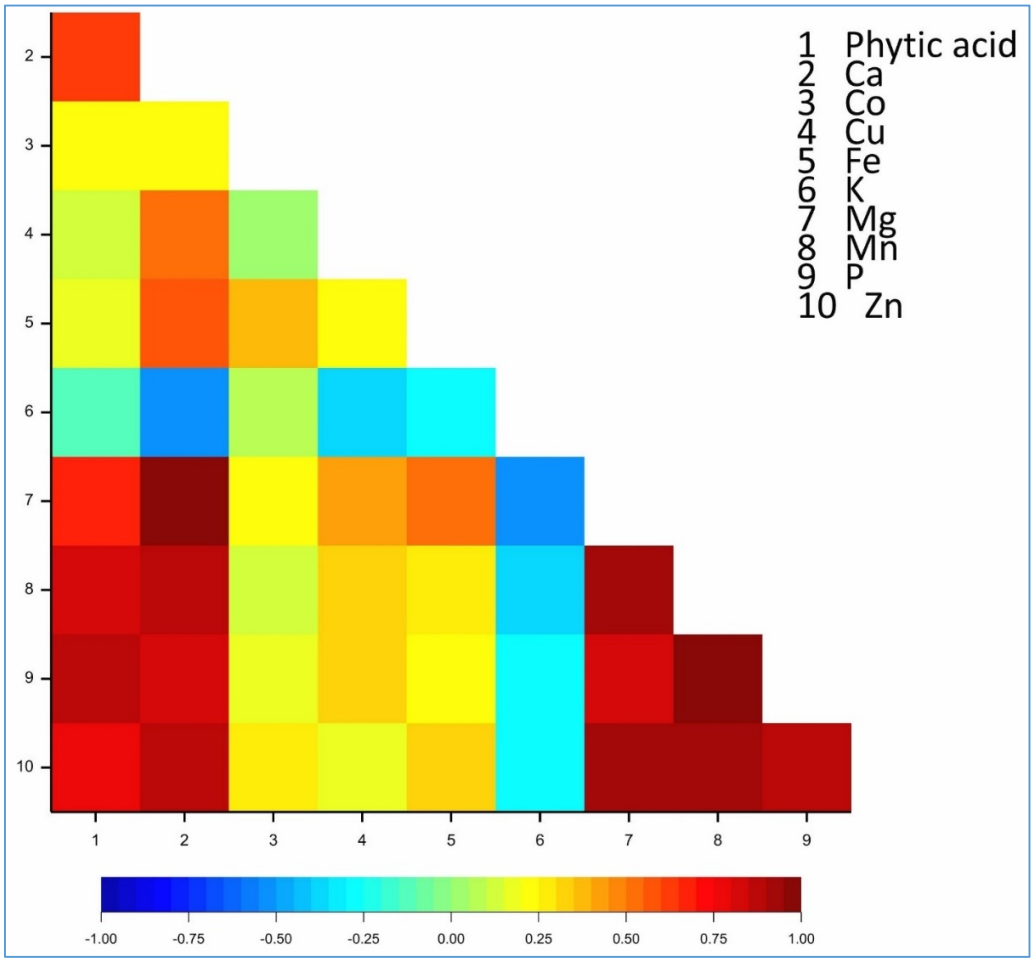



Figure 2-15 Relationship of wheat leaf Phytic acid concentration (mg $kg^{-1}$) with other leaf mineral elements in a hydroponic system with four levels of phosphorus. Data corresponds to three years. Colour corresponds to the strength and direction of the correlation from strongly negative (dark blue) to strongly positive (dark red). Ca (n=67), Co (n=50), Cu (n=67), Fe (n=40), K (n=67), Mg (n=67), Mn (n=67), P (n=67), Zn (n=67), Phytic acid (n=76).

Mineral concentrations were analysed with an unbalanced ANOVA blocking by set and Fisher's unprotected LSD for multiple comparisons in order to understand the effect of the P treatments in wheat leaf, Figure 2-16. Out of nine minerals analysed, two (Co and Fe) were not significantly affected by the P treatments in leaves. Ca, Cu, Mg, Mn, P and Zn mean concentrations tend to increase with the

increasing levels of phosphorus supply, whereas K showed a decreasing tendency. The standard recipe of nutrient solution for hydroponics used in our laboratory is the one given by treatment 3 (Treatments are labelled as: T1=0.625 mmol $L^{-1}$, T2=0.125 mmol $L^{-1}$, T3=0.25 mmol $L^{-1}$, T4=0.5 mmol $L^{-1}$ $KH_2PO_4$). Calcium increased from a mean of 5320 mg $kg^{-1}$ in T3 to 9056 mg $kg^{-1}$ in T4, whereas the two lowest treatments had mean values of 2638 and 2998 mg $kg^{-1}$, T1 and T2 respectively. Copper had mean values of 10.6, 11.23, 12.72 and 13.20 mg $kg^{-1}$ for T1-4 respectively. Magnesium lowest concentrations were for T1 (860 mg $kg^{-1}$) and T2 (921 mg $kg^{-1}$), whereas T3 and T4 had 1716 and 3635 mg $kg^{-1}$, respectively. Manganese concentrations rose from 31, 37.48 and 68.17 mg $kg^{-1}$ in T1, 2 and 3 respectively to 128.68 mg $kg^{-1}$ in T4. Phosphorus concentrations were for T1 to T4 respectively: 1816, 2640, 4443 and 8842 mg $kg^{-1}$. For zinc, the biggest effect was seen on T4 with 31.39 mg $kg^{-1}$, whereas T1, 2 and 3 were 21.37, 20.19 and 17.06 mg $kg^{-1}$. IN the case of potassium we observed a decreasing tendency from T1 42379 mg $kg^{-1}$ to 39252 and 36301 mg $kg^{-1}$ in T2 and T3 to its lowest on T4 with a mean of 33214 mg $kg^{-1}$.

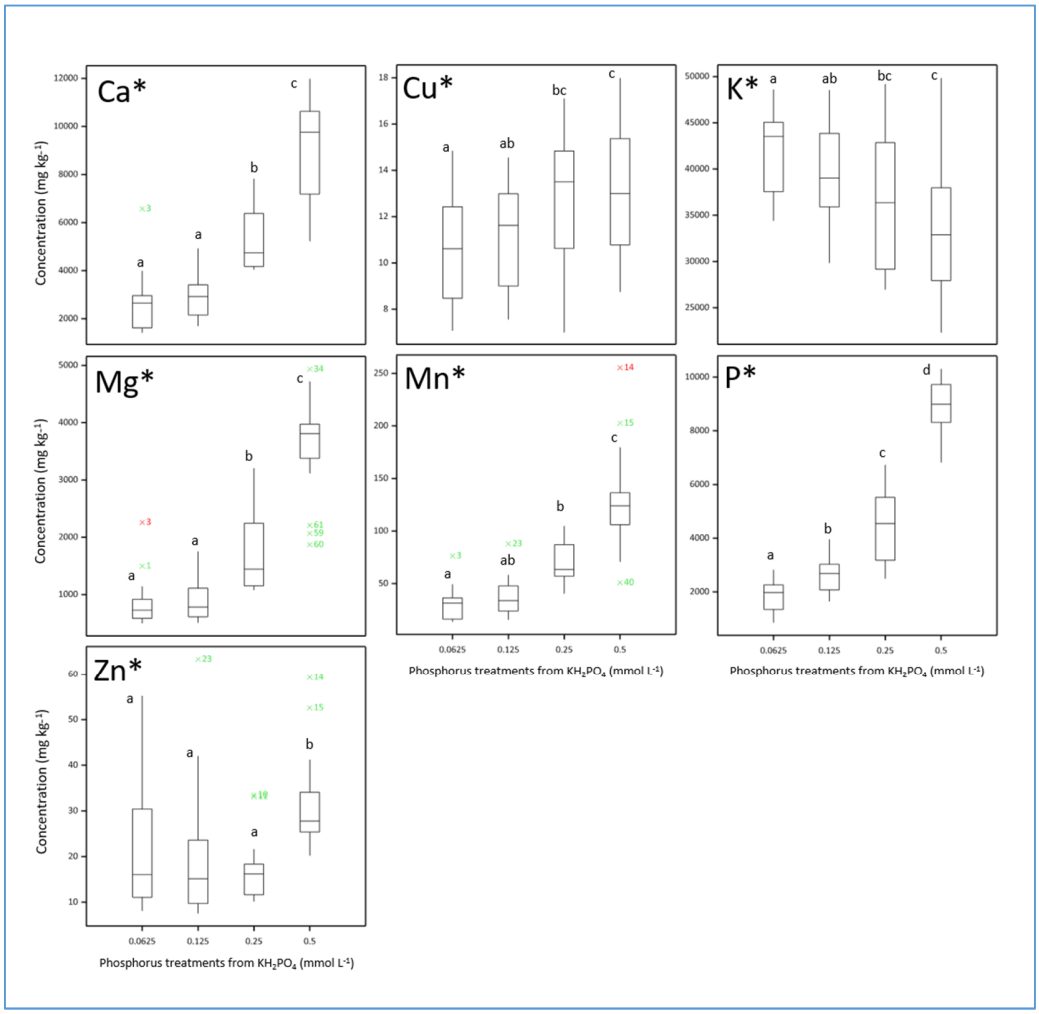



Figure 2-16 Minerals in wheat leaf grown in a hydroponic system as affected by different levels of phosphorus supply (mg $kg^{-1}$). X-axis shows phosphorous treatments from $KH_2PO_4$ (mmol $L^{-1}$) Data corresponds to three years. Boxes followed by a common letter are not significantly different by Unbalanced ANOVA and Fisher's unprotected LSD test at the *p=0.01 level of significance. Total n for each element: Ca, Cu, K, Mg, Mn, P, Zn n=67, Co n=50, Fe n=40. T1=0.0625 mmol $L^{-1}$, T2=0.125 mmol $L^{-1}$, T3=0.25 mmol $L^{-1}$, T4=0.5 mmol $L^{-1}$ $KH_2PO_4$. Schematic boxplot shows the interquartile range with the median drawn; whiskers are 1.5 times the interquartile range beyond the quartiles, or the maximum value if that is smaller. Red crosses are far outliers (three times the interquartile range beyond the quartiles) and green crosses are outliers.

#### *2.4.2.3 PA molar ratios in leaves*

Phytic acid millimolar ratios were calculated dividing the millimoles of PA by the millimoles of each of the mineral elements. PA:Ca, was not significantly affected by the phosphorus input. Whereas Co, Cu, Fe, K and Zn were significantly affected by the treatments (Fisher's LSD, $p<0.001$). In general, the ratios tended to increase with the increasing levels of phosphorus; the only exception was the zinc. Here we discuss the results of two of the most important minerals associated to PA, iron and zinc. Iron PA ratios increased from 0.48 to 2.79 in T1 to T4, and PA:Zn ratios increased from T1 3.48 to 10.98 then dropped to 7.86 in T4. Figure 2-17 shows iron and zinc mean ratios for each of the phosphorus treatments.

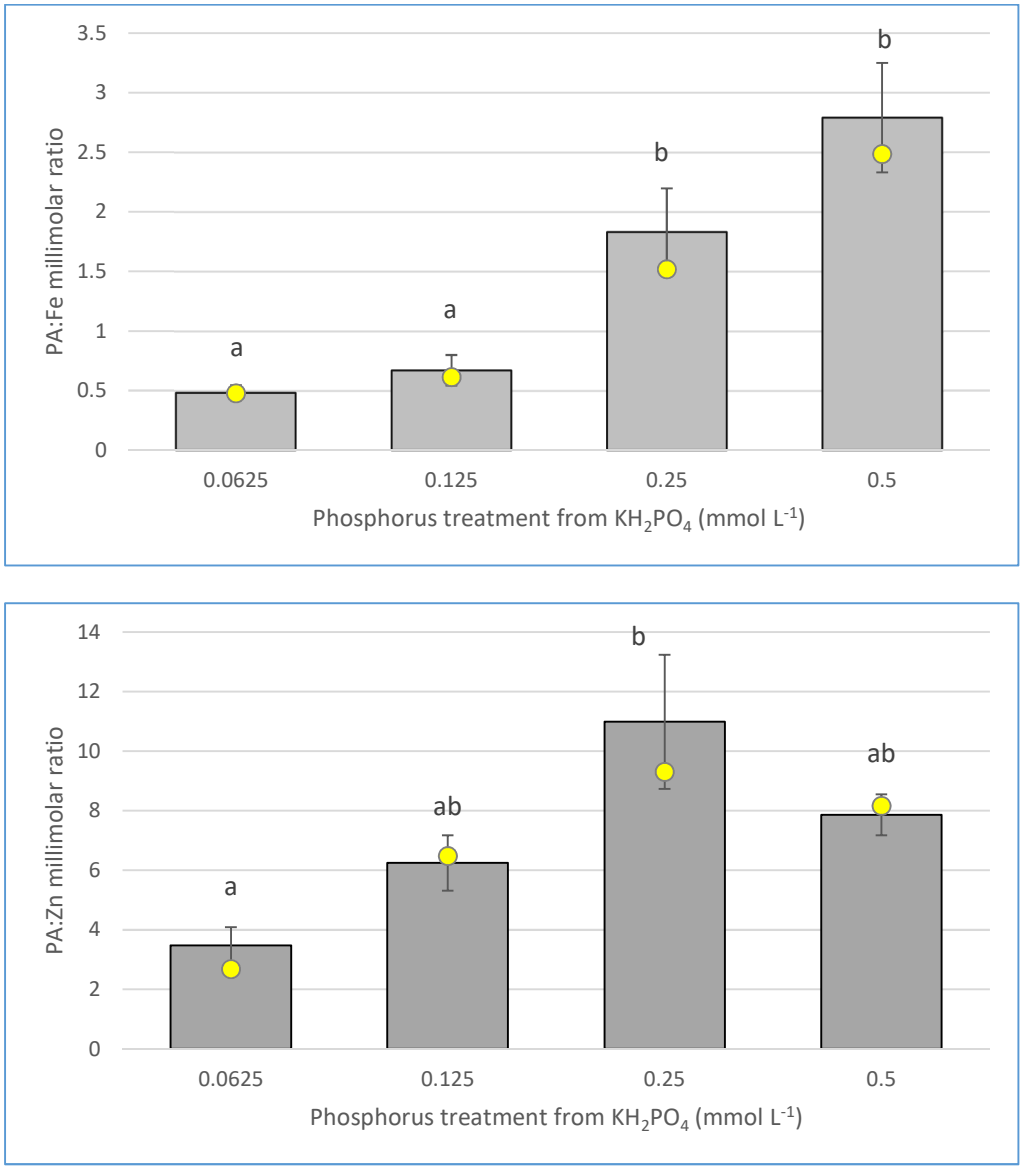



Figure 2-17 Effect of phosphorus treatments over the PA to Fe and Zn ratios in wheat leaves grown in a hydroponic system. Means followed by a common letter are not significantly different by Fisher's unprotected LDS test at the p=0.01 level of significance. Bars are mean ± SE; yellow dots represent the median. T1=0.0625 mmol $L^{-1}$, T2=0.125 mmol $L^{-1}$, T3=0.25 mmol $L^{-1}$, T4=0.5 mmol $L^{-1}$ $KH_2PO_4$. PA:Fe T1, T2, T3, T4 n=10. PA:Zn T1, T2, T3 n=16, T4 n=19.

### 2.4.3 NFT leaf zinc experiment

In total, 72 samples of leaf were analysed in three sets. The phytic acid concentration ranged between 171.9 mg $kg^{-1}$ to 5288 mg $kg^{-1}$. The real minimum value was 603.82 mg $kg^{-1}$, but the values substituted for half-LOD's (2 data points) were also included in the range. Global mean was 2054 ± 944.5 mg $kg^{-1}$ with a median of 2007 mg $kg^{-1}$. Means of each set were, 1764 ± 1101, 1580 ± 429.7 and 2794 ± 588 mg $kg^{-1}$ for Set One, Two and Three respectively (mean ± SD).

LODs and LOQs used can be consulted in Table 9-14. Two values were substituted for half-LODs, both from Set One and corresponded to one data point in treatment 1 and one data point in treatment 2. No values were higher than the calculated LOQ, for calculation details see Table 9-14.

Unbalanced ANOVA analysis showed there were no significant differences between the benches ($p=0.060$), but there were differences between sets ($p<0.001$, LSD 1 %=608) and treatments ($p=0.039$, LSD 5 %=506.4). Therefore the analysis was blocked by set. Sets One and Two were significantly different from Set Three. Table 2-10 shows a summary of statistics by set and bench.

Table 2-10 Phytic acid mean, median and SD by set and bench of leaf samples in an NFT zinc experiment. Data are in mg $kg^{-1}$.

| | | Bench A | | | | Bench B | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Set** | **Zinc tx level** | **n** | **Mean** | **SD** | **Median** | **n** | **Mean** | **SD** | **Median** |
| 1 | 1 | 3 | 1674 | 835 | 2004 | 4 | 684 | 476 | 621 |
| 1 | 2 | 3 | 1058 | 970 | 909 | 4 | 1503 | 425 | 1324 |
| 1 | 3 | 3 | 1863 | 129 | 1809 | 3 | 1465 | 641 | 1449 |
| 1 | 4 | 5 | 3116 | 1464 | 3196 | 2 | 2564 | 675 | 2564 |
| 2 | 1 | 3 | 1550 | 358 | 1526 | 3 | 1330 | 164 | 1406 |
| 2 | 2 | 3 | 2177 | 813 | 2169 | 2 | 1802 | 31 | 1802 |
| 2 | 3 | 3 | 1333 | 183 | 1376 | 3 | 1399 | 302 | 1543 |
| 2 | 4 | 2 | 1731 | 110 | 1731 | 2 | 1372 | 158 | 1372 |
| 3 | 1 | 3 | 2692 | 272 | 2741 | 3 | 2374 | 453 | 2453 |
| 3 | 2 | 3 | 3378 | 509 | 3227 | 3 | 2761 | 387 | 2871 |
| 3 | 3 | 3 | 2707 | 442 | 2729 | 3 | 3433 | 1125 | 2802 |
| 3 | 4 | 3 | 2572 | 222 | 2696 | 3 | 2438 | 310 | 2438 |

tx, treatment; SD, standard deviation; NFT, Nutrient Film Technique; n, number of values.

Unbalanced ANOVA analysis was performed to study the effects of four treatment levels of zinc on the concentration of phytic acid in wheat leaf samples, the analysis was blocked by set. Treatments were not significant if the analysis was not blocked by set. The results showed a significant effect of the treatments ($p<0.05$). Mean values for each treatment were 1663 ± 811.2, 2097 ± 959.5, 2033 ± 939.2 and 2467 ± 968.1 mg $kg^{-1}$, T1 to T4 respectively. Zinc treatments were labelled as: treatment 1 (0.1 µmol $L^{-1}$), treatment 2 (1 µmol $L^{-1}$), treatment 3 (5 µmol $L^{-1}$) and treatment 4 (10 µmol $L^{-1}$). Figure 2-18 top chart.

Phytic acid-P/total P % values were very similar to those observed in the phosphorus experiment. There were no significant differences between the treatments, mean values were 7.7, 9.8, 9.9 and 10.3 %, T1 to T4 respectively. Figure 2-18, bottom chart.

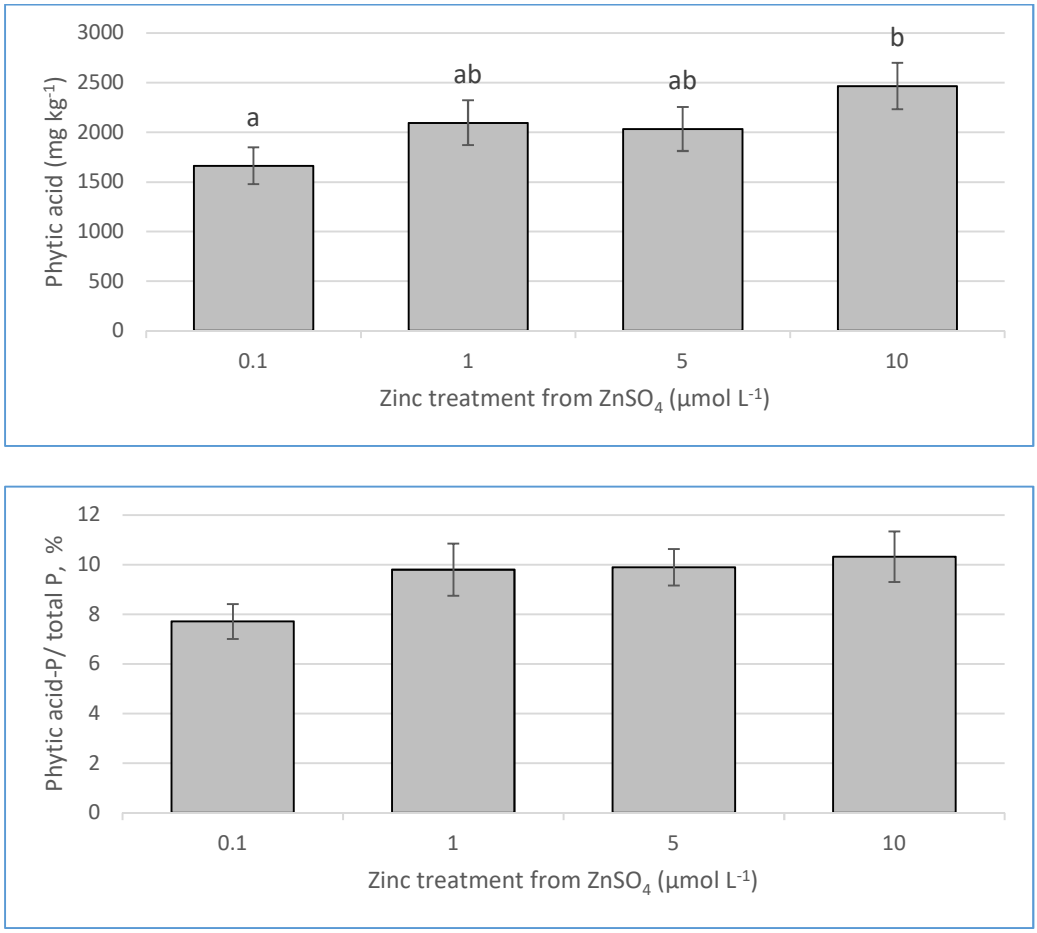



Figure 2-18 Phytic acid concentration in leaf samples by treatment applied in the hydroponic system. Three-year data. Top chart, mean values ± standard error bars (SE). Means followed by a common letter are not significantly different by Fisher's unprotected LDS test at the p=0.05 level of significance. T1 n=19, T2 n=18, T3 n=18, T4 n=17. Bottom chart, Phytic acid-P/total P %, means ± SE bars. T1=0.1 µmol $L^{-1}$, T2=1.0 µmol $L^{-1}$, T3=5.0 µmol $L^{-1}$, T4=10.0 µmol $L^{-1}$ $ZnSO_4$. T1 n=18, T2 n=17, T3 n=18, T4 n=15. Zinc treatment concentration is expressed as micromole per litre (µmol $L^{-1}$).

Data was not following a normal distribution (Shapiro Wilk test, $p<0.001$), after doing the normality tests, mentioned in the previous section to each of the treatments individually and removed two "outliers", data was normalised. New unbalanced ANOVA test was conducted and found set ($p<0.001$) and bench ($p=0.024$) were significant, hence the model was blocked by set and bench. Treatments were only just significant ($p=0.049$). Fisher's unprotected LSD test for multiple comparisons was no different as that from the previous outcome; mean values were 1689 (a), 2095 (ab), 1908 (ab) and 2225 (b) mg $kg^{-1}$ for T1 to T4 respectively. Letters in parenthesis are the result of LSD test, means followed by the same letter were not significantly different at the $p<0.05$ value.

To investigate the effect of the treatments on the zinc concentration in leaf samples an unbalanced ANOVA was conducted blocked by set. Mean zinc concentrations by treatment were: 13.47 ± 2.9, 16.55 ± 5.17, 27.85 ± 6.3 and 42.39 ± 11.26 mg $kg^{-1}$ (mean ± SD). The results of the analysis of variance showed that the treatments ($p<0.001$, LSD 1 %=5.96) and the set ($p=0.002$) were significant but their interaction was not significant. Fisher's LSD test showed that means of treatments 1 and 2 were not significantly different, whereas means from treatment 3 were different from the rest and similarly, means from treatment 4 were different from the rest of the treatments, Figure 2-19.

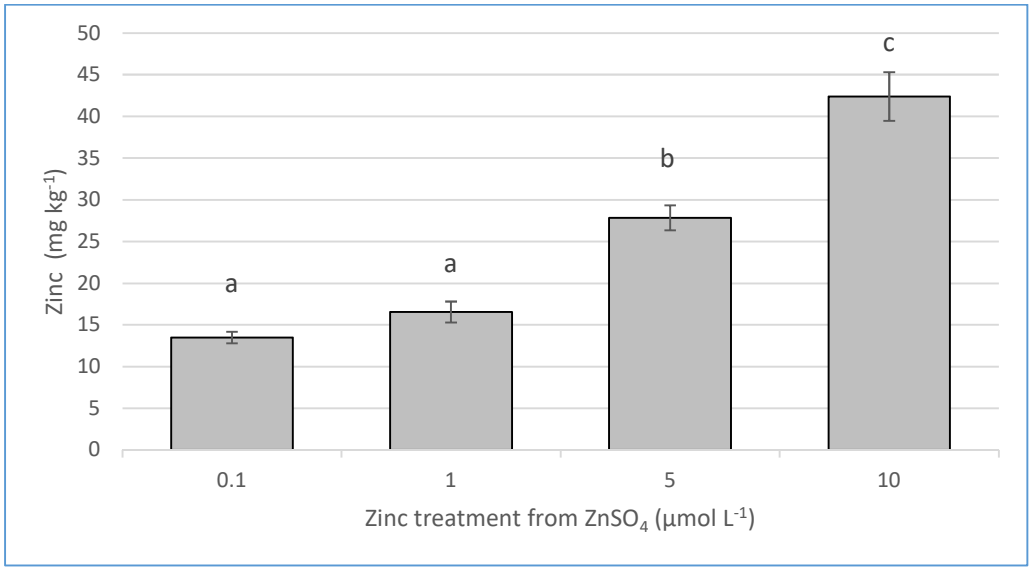



Figure 2-19 Zinc concentration in leaf samples by treatment applied in the hydroponic system. Mean values ± standard error bars (SE). Means followed by a common letter are not significantly different by Fisher's unprotected LDS test at the p=0.01 level of significance. Three-year data. T1=0.1 µmol $L^{-1}$, T2=1.0 µmol $L^{-1}$, T3=5.0 µmol $L^{-1}$, T4=10.0 µmol $L^{-1}$ $ZnSO_4$. T1 n=18, T2 n=17, T3 n=18, T4 n=15.

### *2.4.3.1 Phytic acid and mineral interactions*

There were no significant relationship of phytic acid concentrations and other minerals in leaf. Nevertheless, it was observed that Zn concentrations showed a strong negative relationship with the concentration of Cu (r= -0.684, p<0.001). Calcium showed significant medium relationship with Cu (r= 0.568 p=0.004), K (r= -0.556 p=0.005), Mg (r= 0.877 p<0.001), and Mn (r= 0.550 p=0.005), Co and Fe (r=0.778, p<0.001). Interestingly, in this experiment, P was not associated to phytic acid (r=0.184, p=0.39); K was the only element significantly linked with P (r= 0.840 p<0.001) Figure 2-20.

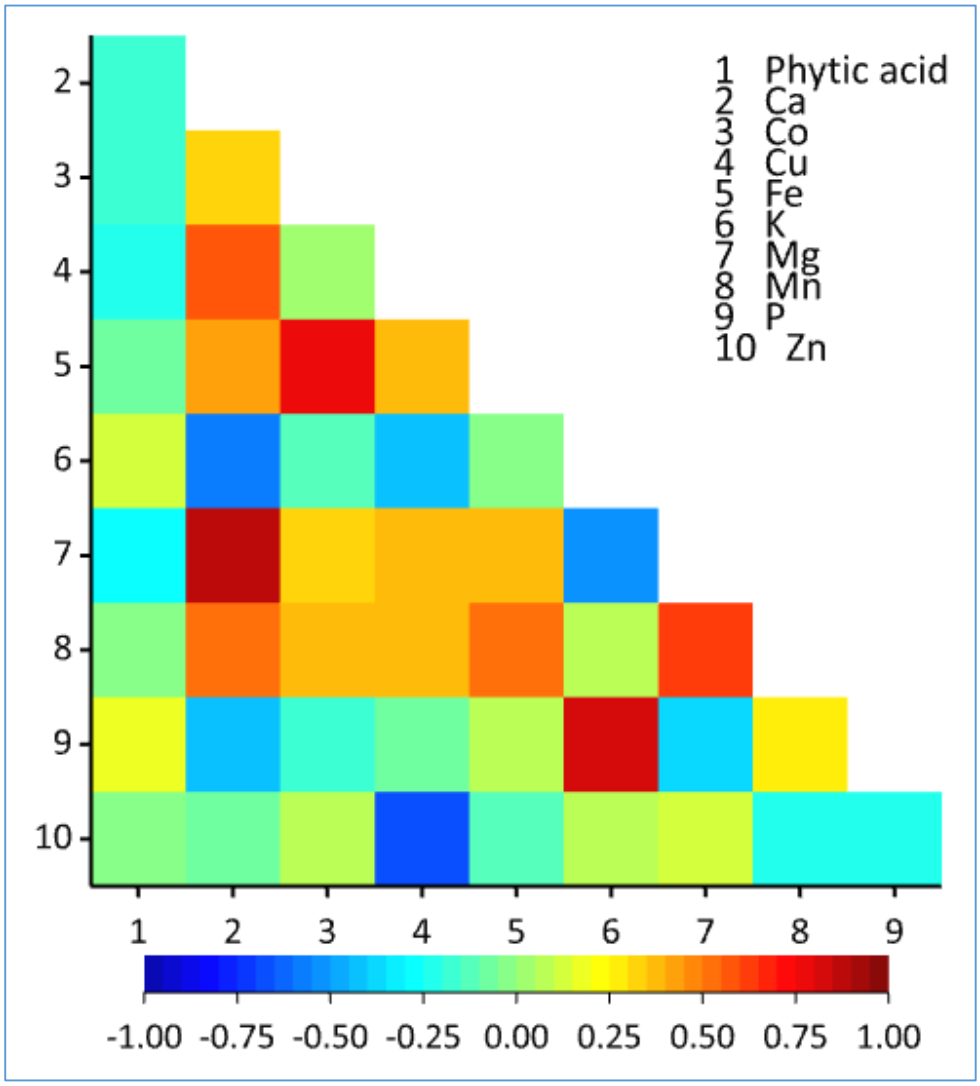



Figure 2-20 Relationship of wheat leaf phytic acid concentration (mg $kg^{-1}$) with other leaf mineral elements in a hydroponic system with four levels of zinc. Data corresponds to three years. Colour corresponds to the strength and direction of the correlation from strongly negative (dark blue) to strongly positive (dark red). Ca (n=68), Co (n=45), Cu (n=68), Fe (n=47), K (n=68), Mg (n=68), Mn (n=68), P (n=68), Zn (n=68), Phytic acid (n=72).

#### *2.4.3.2 Effect of zinc treatments on mineral concentrations*

Mineral concentrations were analysed with unbalanced ANOVA blocked by set and Fisher's unprotected LSD test to investigate the effect of the Zn supply treatments in wheat leaf minerals. Out of nine minerals analysed, seven were

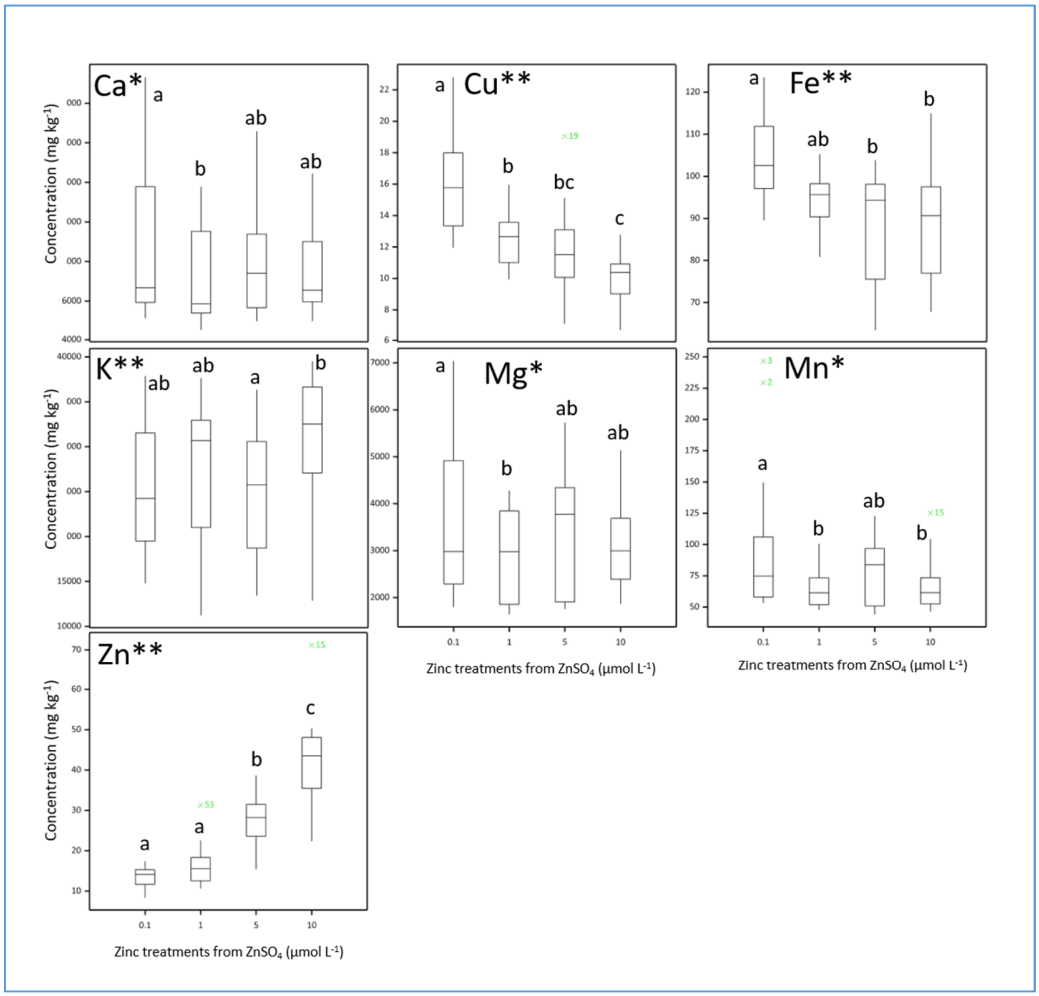
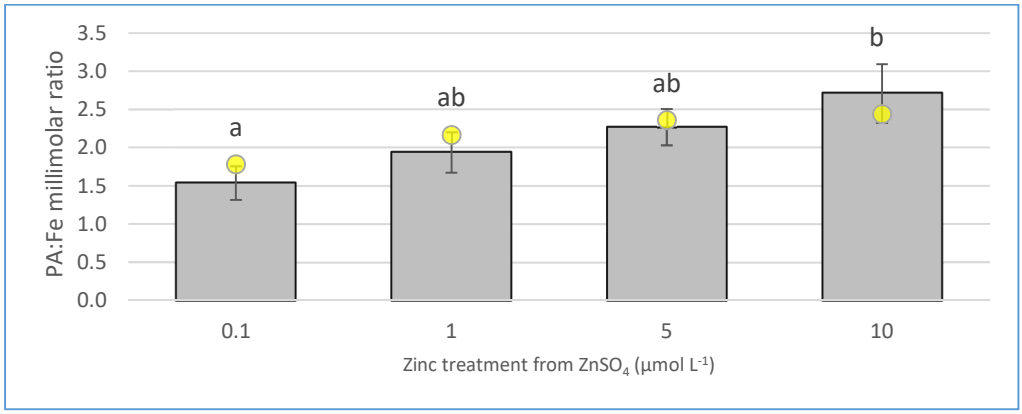


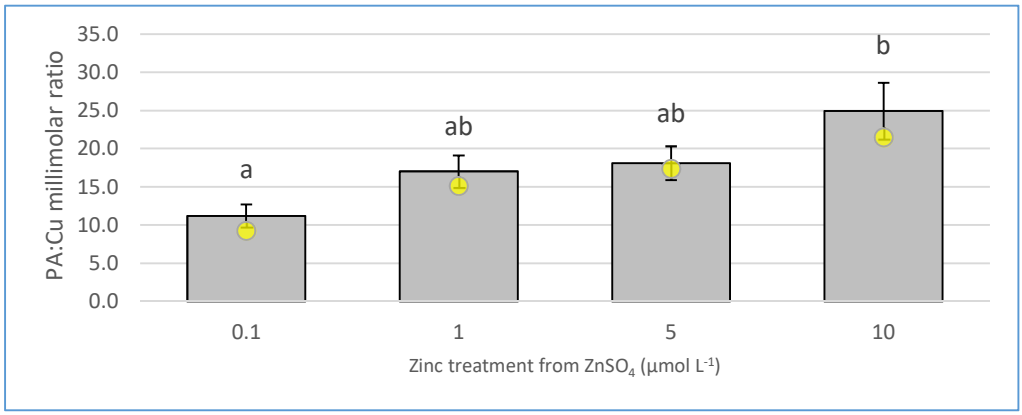

Figure 2-21 Mineral concentrations (mg $kg^{-1}$) in wheat leaves grown in a hydroponic system as affected by different levels of zinc supply. X-axis shows zinc treatments from $ZnSO_4$ (µmol $L^{-1}$). Data corresponds to three years. Means followed by a common letter are not significantly different by Fisher's unprotected LDS test at the *p=0.05 and **p=0.01 level. T1=0.1 µmol $L^{-1}$, T2=1.0 µmol $L^{-1}$, T3=5.0 µmol $L^{-1}$, T4=10.0 µmol $L^{-1}$ $ZnSO_4$. Total n for each element: Ca, Cu, K, Mg, Mn, Zn n=68, Fe n=47. Schematic boxplot shows the interquartile range with the median drawn; whiskers are 1.5 times the interquartile range beyond the quartiles, or the maximum value if that is smaller. Green crosses are outliers.

significantly affected by the treatments: Ca (p=0.013, LSD 5 %=1064), Cu (p<0.001, LSD 1 %=1.970), Fe (p=0.001, LSD 1 %=11.48), K (p=0.007, LSD 1 %=3658), Mg

(p=0.040, LSD 5 %=497.1), Mn (p=0.023, LSD 5 %=20.97) and Zn as it was shown previously on Figure 2-19 (p<0.001, LSD 1 %=4.481); Figure 2-21.

Phytic acid millimolar ratios were calculated dividing the millimoles of PA by the millimoles of each one of the mineral elements. Unbalanced ANOVA blocked by set and Fisher's unprotected LSD test showed that PA:Cu (p=0.001, LSD 1 %= 7.978), PA:Fe (p=0.032, LSD 5 %=0.769) and PA:Zn (p<0.001, LSD 1 %=4.15) ratios were significantly affected by the zinc treatments. PA:Cu displayed an increasing tendency, mean ratios were 11.2, 17.0, 18.1 and 25, with medians of 9.2, 15.1, 17.4 and 21.5 for treatments 1 to 4 respectively. PA:Fe also showed an increasing tendency, with mean values of 1.5, 1.9, 2.3 and 2.7 with medians of 1.8, 2.2, 2.4, 2.4. On the contrary, PA:Zn ratios increased with the first two treatments and declined with the two highest treatments, the mean values were, 12.8, 13.5, 7.5, 6.1 with medians of 13.9, 14.6, 7.2 and 5.2, Figure 2-22.



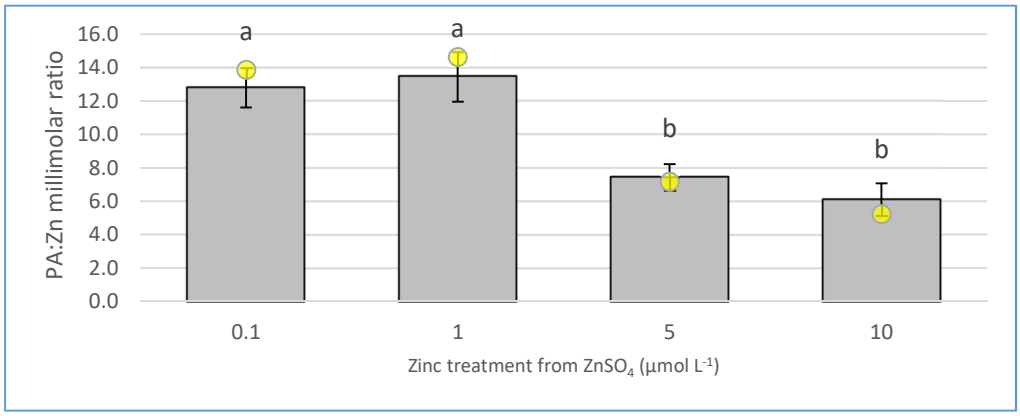




Figure 2-22 Effect of zinc treatments over the PA:Cu, PA:Fe and PA:Zn ratios in wheat leaves grown in a hydroponic system. Means followed by a common letter are not significantly different by Fisher's unprotected LSD test at the p=0.05 (PA:Fe) and p=0.01 (PA:Cu, PA:Zn) level of significance. Bars are means ± SE bars; yellow dots represent the median. T1=0.1 µmol $L^{-1}$, T2=1.0 µmol $L^{-1}$, T3=5.0 µmol $L^{-1}$, T4=10.0 µmol $L^{-1}$. PA:Cu, PA:Zn T1, T3 n=18, T2 n=17, T4 n=15; PA:Zn T1, T2, T3 n=12, T4 n=11; PA:Zn.

### 2.4.4 NFT grain phosphorus experiment

In total, 72 samples of wholegrain were analysed in three sets. The phytic acid concentration ranged between 5948 and 28178 mg $kg^{-1}$, a global mean of 13737 ± 3781 mg $kg^{-1}$, median of 13146 mg $kg^{-1}$. Medians for each set were: 16016.03, 12047.13 and 12405.28 mg $kg^{-1}$ for sets One, Two and Three respectively. Mean values for sets One, Two and Three were, respectively: 15356.72 ± 4322, 12493.28 ± 3853 and 13784.77 ± 2737 mg $kg^{-1}$ (mean ± SD).

LODs and LOQs used for each individual set can be consulted in Table 9-14. No data points were lower than the corresponding LOD, therefore no data was substituted. Similarly, no values were higher than the calculated LOQ.

An unbalanced ANOVA test was used to analyse if the phosphorus treatments had an effect on the PA concentrations in wheat grain grown in a hydroponic system. The results showed the bench had no significant effect. Whereas phosphorus treatments had a significant effect ($p<0.001$ LSD 1 %=2238) and the set was also significant ($p=0.001$). Therefore, the analysis was blocked by sets. Table 2-11 shows a summary of statistics by set and bench.

Table 2-11 Phytic acid mean, median and SD by set and bench of grain samples in an NFT Phosphorus experiment. Data are in mg $kg^{-1}$.

| | | Bench A | | | | Bench B | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Set** | **Phosphorus tx level** | **n** | **Mean** | **SD** | **Median** | **n** | **Mean** | **SD** | **Median** |
| 1 | 1 | 2 | 10355 | 2799 | 10355 | 3 | 10221 | 1403 | 10491 |
| 1 | 2 | 3 | 17083 | 857 | 17371 | 3 | 18749 | 8209 | 14870 |
| 1 | 3 | 2 | 16767 | 915 | 16767 | 3 | 15727 | 1236 | 16148 |
| 1 | 4 | 2 | 18149 | 3130 | 18149 | 2 | 15627 | 664 | 15627 |
| 2 | 1 | 3 | 6585 | 945 | 6137 | 3 | 9775 | 581 | 9621 |
| 2 | 2 | 3 | 9946 | 34 | 9935 | 3 | 9567 | 591 | 9637 |
| 2 | 3 | 3 | 13180 | 60 | 13159 | 3 | 12206 | 678 | 12047 |
| 2 | 4 | 3 | 17723 | 689 | 17853 | 6 | 16729 | 1877 | 16078 |
| 3 | 1 | 4 | 11974 | 418 | 11992 | 3 | 12666 | 723 | 12405 |
| 3 | 2 | 3 | 12672 | 410 | 12623 | 3 | 11138 | 698 | 11038 |
| 3 | 3 | 3 | 13368 | 2136 | 12237 | 3 | 13223 | 1823 | 14152 |
| 3 | 4 | 3 | 16578 | 294 | 16429 | 3 | 19262 | 1500 | 18895 |

tx, treatment; SD, Standard Deviation; NFT, Nutrient Film Technique; n, number of values.

Phosphorus treatments were labelled as treatment 1 (0.0625 mmol $L^{-1}$), treatment 2 (0.125 mmol $L^{-1}$), treatment 3 (0.25 mmol $L^{-1}$) and treatment 4 (0.5 mmol $L^{-1}$). Fisher's unprotected LSD at the p=0.01 level defined three clusters, the extreme treatments were significantly different and showed an increasing tendency along the P supply. Treatment 1 had a mean PA of 10353 ± 2249 mg $kg^{-1}$ (mean ± SD) whereas in treatment 4 the PA concentration increased 17295 ± 1766 mg $kg^{-1}$ (mean ± SD), Figure 2-23 top chart.

Because we found that the residuals were not following a normal distribution according to the W-test, we also ran a Kruskal-Wallis one-way ANOVA, the results

also showed that the treatments were significantly affecting the concentrations of PA in grain (Chi square <0.001). The mean ranks were 17.11, 30.17, 38.94 and 58.68 for T1 to T4 respectively.

Phytic acid-P/total P %, was 64.03 %, 66.62 %, 60.72 % and 63.03 % for treatments 1, 2, 3 and 4 respectively, Figure 2-23 bottom chart. For this calculation, the phytic acid-P determined by the Megazyme Kit is compared to the total P which is obtained by ICP-MS and then expressed as a percentage. There were no significant differences between the treatments (p=0.230) but the sets were significantly different (p<0.001). Each set was significantly different from the other two (Fisher's unprotected LSD 1 %= 7.21). Mean values for each set were: 73.3 %, 56.5 % and 65 % for set One, Two and Three respectively.

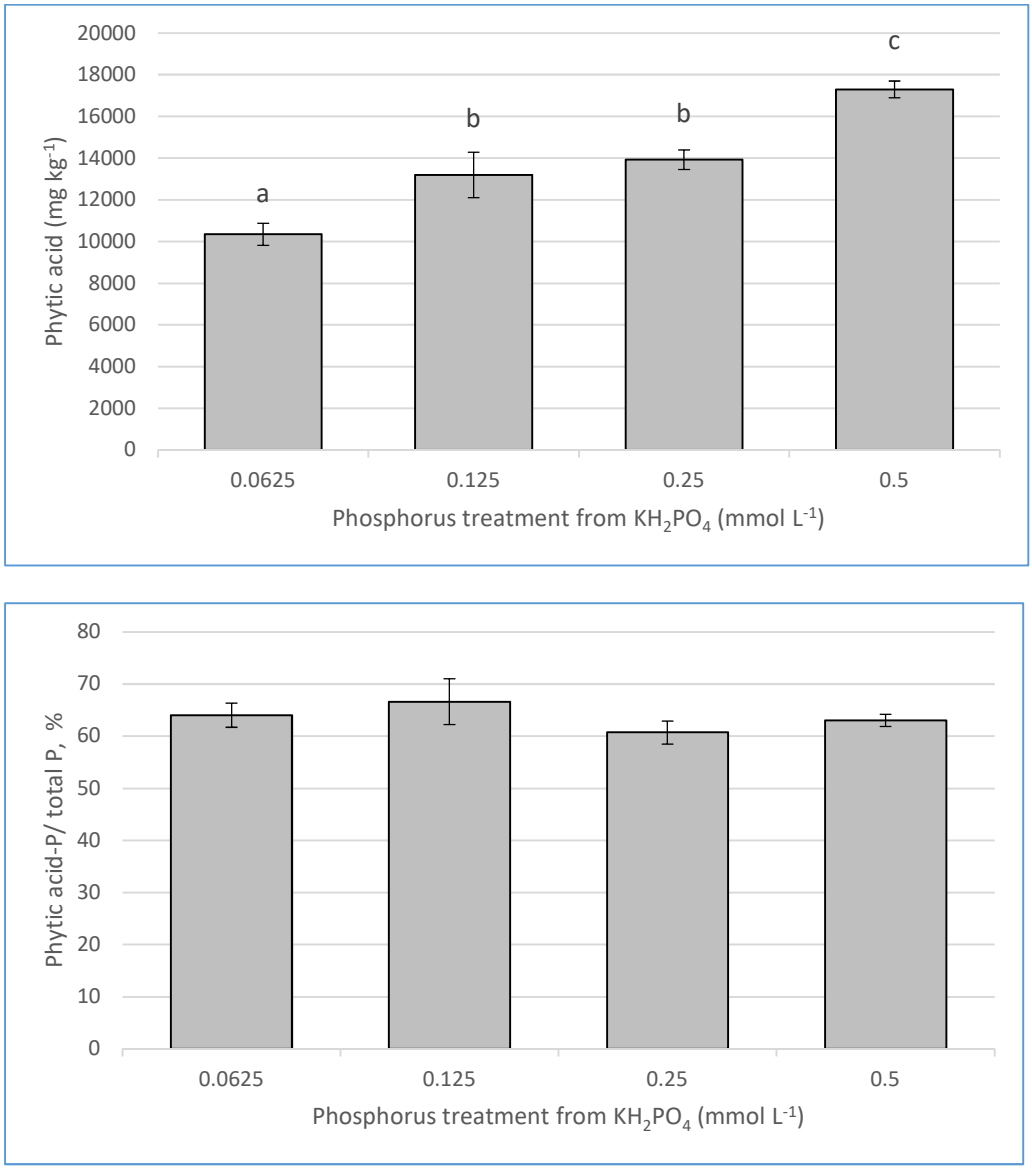



Figure 2-23 Phytic acid concentration in grain samples by treatment applied in the hydroponic system. Three year data. Top chart, mean values ± standard error bars (SE). Means followed by the same letter are not significantly different at the p=0.01 level (unbalanced ANOVA and Fisher's unprotected LSD test). T1-T2 n=18, T3 n=17, T4 n=19. Bottom chart, Phytic acid-P/total P %, mean ± SE bars. T1 n=17, T2-T3 n=16, T4 n=19. Phosphorus treatment concentrations are expressed as millimole per litre (mmol $L^{-1}$). T1=0.0625 mmol $L^{-1}$, T2=0.125 mmol $L^{-1}$, T3=0.25 mmol $L^{-1}$, T4=0.5 mmol $L^{-1}$ $KH_2PO_4$.

Grain yield (GYD) expressed as grams per plant, was significantly affected by the phosphorus treatments. Unbalanced ANOVA and Fisher's unprotected LSD ($p<0.001$, LSD 1 %= 1.931), blocking by set and bench showed the grain yield increased as the P concentration was increased in the nutrient solution. There were no significant differences between the benches and/or the sets, as main effects. Grain yield mean values were 8.22 ± 2, 11.06 ± 3.89, 19.33 ± 8.25 and 15.56 ± 8.05 g per plant (mean ± SD) for T1-T4 respectively, Figure 2-24.

The number of ears per plant were counted and also analysed with an unbalanced ANOVA blocked by set. The results showed that the phosphorus treatments had a significant effect over the number of ears per plant ($p<0.001$). Fisher's LSD unprotected test showed treatments 1 and 2 (7.6 and 10.7 ears per plant) were significantly different from treatments 3 and 4 (15.6 and 17.6), LSD 1 %= 1.586.

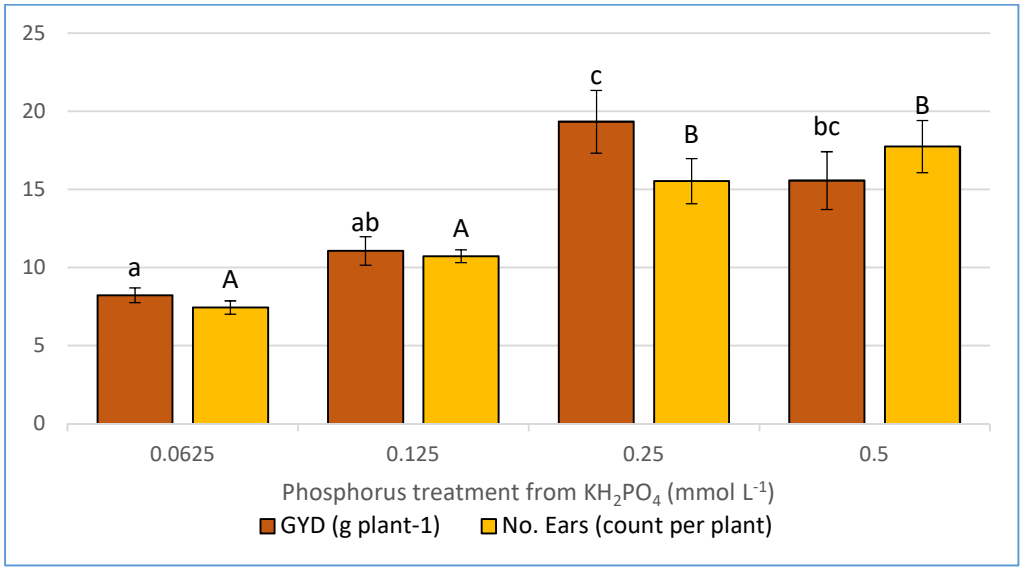



Figure 2-24 Grain yield (GYD, g plant$^{-1}$) and number of ears per plant of wheat grown in a hydroponic system with four levels of phosphorus. Means ± SE bars. Data corresponds to three years. Means followed by the same letter are not significantly different at the p=0.01 level, unbalanced ANOVA and Fisher's unprotected LSD; lower case letters are differences between treatments for GYD; capital letters are differences between treatments for number of ears. X-axis are phosphorus treatments, T1=0.0625 mmol L$^{-1}$, T2=0.125 mmol L$^{-1}$, T3=0.25 mmol L$^{-1}$, T4=0.5 mmol L$^{-1}$ $KH_2PO_4$. T1, 2 n=18, T3 n=17, T4 n=19.

### *2.4.4.1 Phytic acid and mineral interactions*

Phytic acid concentration showed a strong positive significant relationship with wholegrain mineral concentrations; Cu (r=0.871, p<0.001), Fe (r=0.651, p<0.001), K (r=0.692, p<0.001), Mg (r=0.797, p<0.001), P (r=0.899, p<0.001), Zn (r=0.644, p<0.001). Calcium and manganese also had a significant but rather weak relationship with phytic acid, Ca ($r^2$=0.402, p=0.003), Mn ($r^2$=0.329, p=0.017), Figure 2-25.

To investigate if a linear equation could describe the PA levels in wholegrain based on the concentrations of P; a simple linear regression was conducted. The

regression was significant ($p<0.001$) and it was described by the equation $y= 2.165x + 536$ and it accounted for 62.3 % of the variance observed.

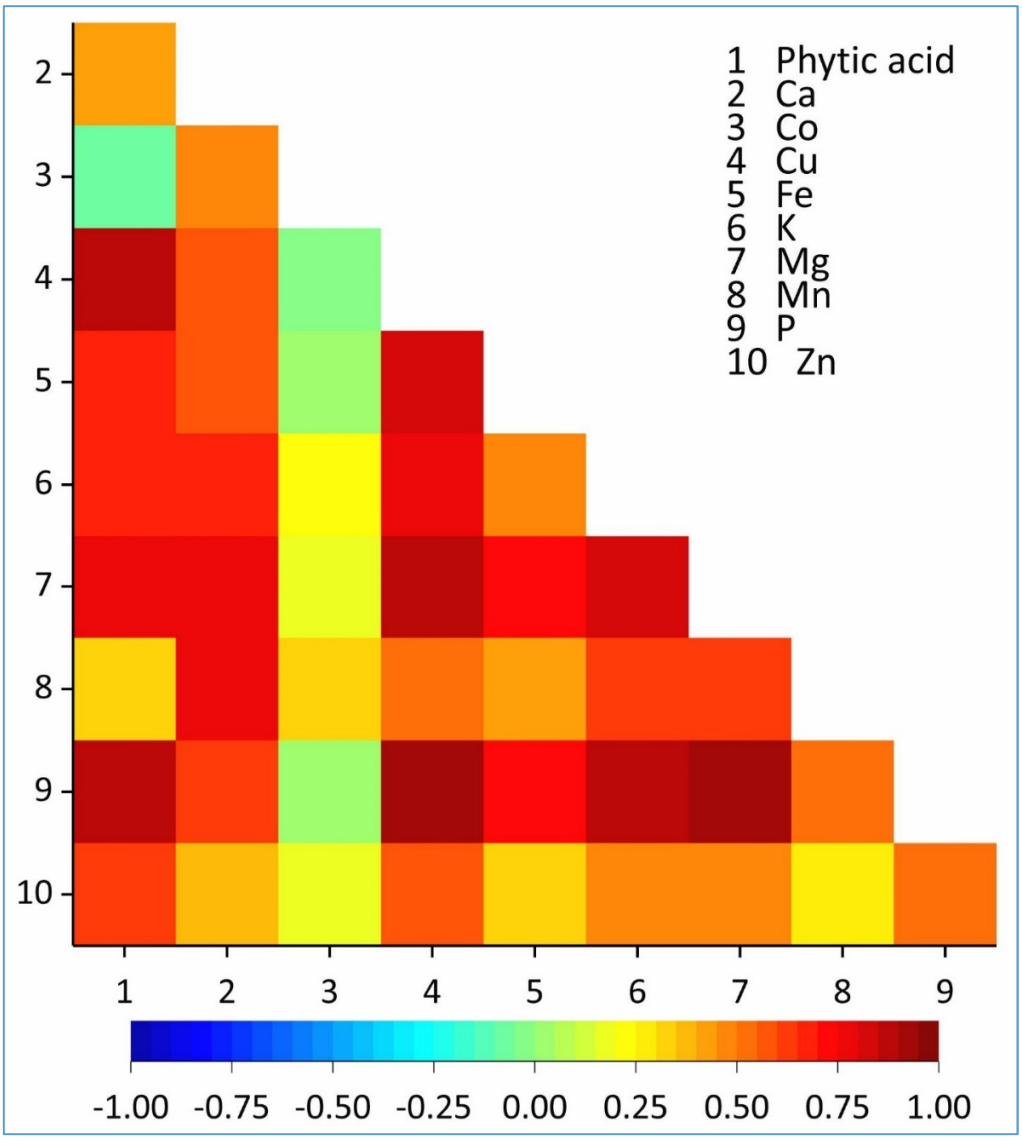



Figure 2-25 Relationship of wheat grain phytic acid concentration (mg $kg^{-1}$) with other mineral elements in a hydroponic system with four levels of phosphorus. Data corresponds to three years. Colour corresponds to the strength and direction of the correlation from strongly negative (dark blue) to strongly positive (dark red). Ca, Cu, Fe, K, Mg, Mn, P, Zn (n=68), Co (n=52), phytic acid (n=72).

#### *2.4.4.2 Effect of phosphorus treatments on grain mineral concentrations*

Mineral concentrations were analysed with an unbalanced ANOVA blocked by set and Fisher's unprotected LSD test to understand the effect of the phosphorus treatments on the accumulation of different minerals in wheat grain, Figure 2-26. Eight out of nine minerals analysed were affected significantly ($p<0.001$) by the treatments (T1=0.0625 mmol $L^{-1}$, T2=0.125 mmol $L^{-1}$, T3=0.25 mmol $L^{-1}$, T4=0.5 mmol $L^{-1}$ $KH_2PO_4$). All minerals exhibited an increase in concentration with the increasing phosphorus supply. Calcium increased from 473.17, 448.89 mg $kg^{-1}$ in T1 and T2 to 558.53, 574.80 mg $kg^{-1}$ in T3 and T4 (LSD 1 %=65.41). Copper increased from 8.1, 7.9 and 10.34 mg $kg^{-1}$ in T1, T2 and T3 respectively to 13.51 mg kg in T4 (LSD 1 %= 1.398). Iron showed a big jump from 53.12, 46.79, 50.19 (T1, 2 and 3 respectively) to 73.17 mg $kg^{-1}$ in T4 (LSD 1 %= 16.76). For K, the biggest difference was in T1 to T4 with 6852.36 to 9270.28 mg $kg^{-1}$ in T4 (LSD 1 %= 882.5), similarly Mg biggest increase was from T1 to T4 with 1045.72 to 2207.27 mg $kg^{-1}$ (LSD 1 %= 300.3). Manganese had modest changes 36.53, 35.95, 43.87 and 43.13 mg $kg^{-1}$ in T1-4, respectively (LSD 1 %= 6.178). Phosphorus increased from 4502.81, 5435.78, 6467.05 in T1-3 respectively to 7761.83 in T4 (LSD 1 %= 618.5), and finally, zinc had mean concentrations of 32.03, 23.35, 30.75 mg $kg^{-1}$ in T1-3 respectively and increased to 41.77 mg $kg^{-1}$ in T4. Data mentioned corresponds to mean values of all sets.

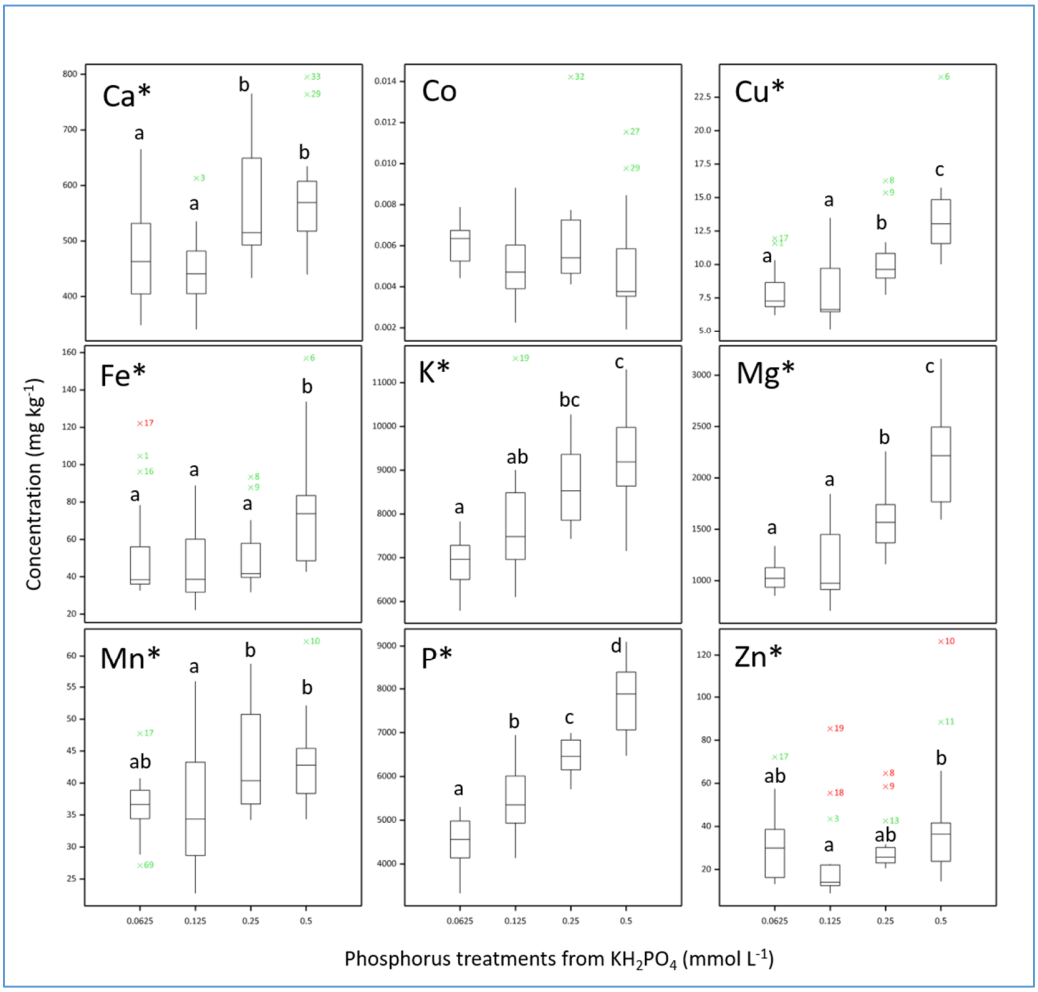



Figure 2-26 Mineral concentrations (mg $kg^{-1}$) in wheat grain grown in a hydroponic system as affected by different levels of phosphorus supply. X-axis shows phosphorus treatments from $KH_2PO_4$ (mmol $L^{-1}$). Data corresponds to three years. Boxes followed by the same letter are not significantly different at the *p=0.01 level (Unbalanced ANOVA, blocked by set and Fisher's unprotected LSD test). T1=0.0625 mmol $L^{-1}$, T2=0.125 mmol $L^{-1}$, T3=0.25 mmol $L^{-1}$, T4=0.5 mmol $L^{-1}$ $KH_2PO_4$. Total n for each element: Ca, Cu, Fe, K, Mg, Mn, P, Zn n=68, Co n=52. Schematic boxplot shows the interquartile range with the median drawn; whiskers are 1.5 times the interquartile range beyond the quartiles, or the maximum value if that is smaller. Red crosses are far outliers (three times the interquartile range beyond the quartiles) and green crosses are outliers.

To take into account any possible dilution effect, the concentrations of phytic acid were re-calculated using the grain yield. The phytic acid concentration expressed in mg $kg^{-1}$ were multiplied by the grain yield values (g per plant) and the result was divided by 1000 to obtain PA concentrations in mg per plant.

Phytic acid in mg $plant^{-1}$ was set as the independent variable and the bench, set and treatment were used as factors in an unbalanced ANOVA analysis. The results showed that the bench ($p=0.008$) and the treatments ($p<0.001$) had a significant effect on the phytic acid concentration. Therefore, the bench factor was used for blocking the analysis. Table 2-12 shows a summary of phytic acid means by set and bench.

Table 2-12 Phytic acid mean, median and standard error by set and bench of wheat grain samples in an NFT phosphorus experiment. Data recalculated using the grain yield. Data are in mg $plant^{-1}$.

| | | Bench A | | | | Bench B | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Set** | **Phosphorus Treatment level** | **n** | **Mean** | **SD** | **Median** | **n** | **Mean** | **SD** | **Median** |
| 1 | 1 | 2 | 113.0 | 1.27 | 113.0 | 3 | 85.9 | 20.96 | 78.5 |
| 1 | 2 | 3 | 124.7 | 11.11 | 125.1 | 3 | 108.0 | 2.08 | 109.1 |
| 1 | 3 | 2 | 312.4 | 36.21 | 312.4 | 3 | 170.9 | 97.73 | 175.2 |
| 1 | 4 | 2 | 281.4 | 170.44 | 281.4 | 2 | 326.0 | 105.48 | 326.0 |
| 2 | 1 | 3 | 47.6 | 10.83 | 48.8 | 3 | 88.9 | 17.44 | 94.4 |
| 2 | 2 | 3 | 103.4 | 15.01 | 110.0 | 3 | 134.0 | 36.02 | 135.9 |
| 2 | 3 | 3 | 443.9 | 44.19 | 461.1 | 3 | 236.2 | 50.80 | 224.4 |
| 2 | 4 | 3 | 502.6 | 112.15 | 534.7 | 6 | 163.9 | 50.15 | 159.6 |
| 3 | 1 | 4 | 88.3 | 16.03 | 92.6 | 3 | 90.1 | 14.12 | 86.0 |
| 3 | 2 | 3 | 156.6 | 14.28 | 161.7 | 3 | 177.5 | 22.06 | 170.2 |
| 3 | 3 | 3 | 225.5 | 20.02 | 234.3 | 3 | 207.9 | 57.82 | 193.8 |
| 3 | 4 | 3 | 247.8 | 6.16 | 246.5 | 3 | 197.5 | 60.50 | 162.9 |

n=number of values; SD=standard deviation; NFT; Nutrient Film Technique.

Phosphorus treatments had a highly significant effect on the phytic acid concentration ($p<0.001$, LSD 1 % =74.45). Treatments 1 and 2 with mean values of 84.25 ± 22.7 and 134.1 ± 31.61 kg per plant of PA respectively were significantly different from treatments 3 and 4 which had a mean of 263.4 ± 106.4 and 265.4 ± 137.1 mg per plant of phytic acid respectively (means ± SD), Figure 2-27.

The recalculated PA concentrations (mg per plant) were compared to the concentrations of other minerals to see if there were any differences between the original data values and the recalculated data. The similarity matrix showed positive relationship between the PA concentration and Ca ($r=0.553$, $p<0.001$), K ($r=0.696$, $p<0.001$), Mg ($r=0.650$, $p<0.001$), and P ($r=0.563$, $p<0.001$); other significant but rather weak relationships were observed in Co ($r=0.391$, $p=0.004$), Cu ($r=0.410$, $p=0.002$), Mn ($r=0.460$, $p=0.001$) and Zn ($r=0.318$, $p=0.022$), Figure 2-28. Table 2-13 presents a comparative of the differences between the original data values and the recalculated data by GYD.

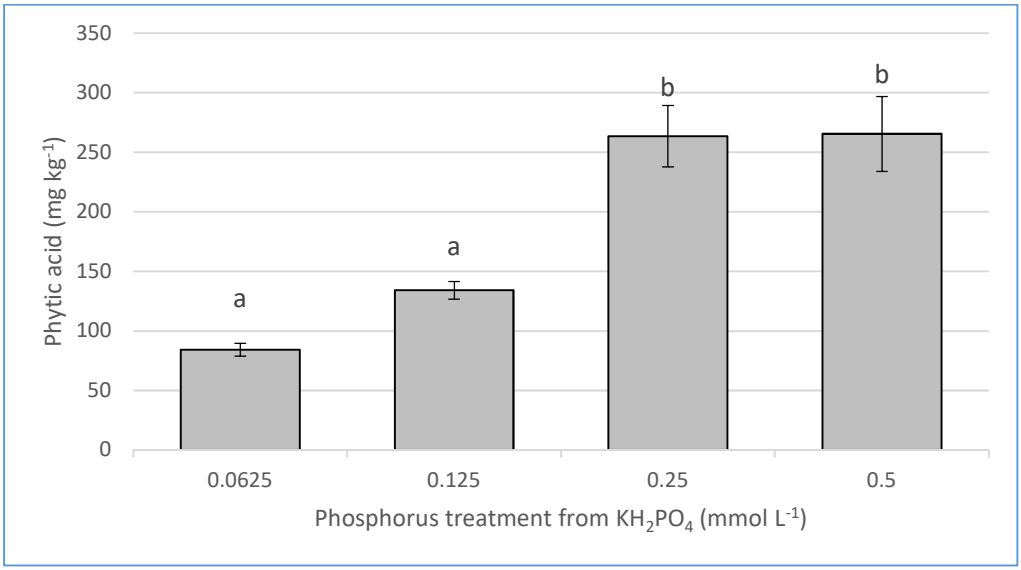



Figure 2-27 Phytic acid content in wholegrain samples by treatment applied in the hydroponic system (mg per plant). Three-year data. Data converted using the grain yield (g per plant). Mean values ± standard error bars (SE). Bars with the same letter are not significantly different at the 1 % probability level (Fisher's test). Treatment 1 (n=18), treatment 2 (n=18), treatment 3 (n=17), treatment 4 (n=19).

Table 2-13 Comparative of correlation coefficients between the original data values and the data recalculated by GYD.

| | PA mg plant$^{-1}$ | | PA mg kg$^{-1}$ | |
|---|---|---|---|---|
| **Ca** | 0.553 | *** | 0.402 | ** |
| **Co** | 0.391 | ** | -0.086 | |
| **Cu** | 0.410 | ** | **0.871** | *** |
| **Fe** | 0.105 | | 0.651 | *** |
| **K** | 0.696 | *** | 0.692 | *** |
| **Mg** | 0.650 | *** | **0.797** | *** |
| **Mn** | 0.460 | *** | 0.329 | * |
| **P** | 0.563 | *** | **0.899** | *** |
| **Zn** | 0.318 | * | 0.644 | *** |

Numbers in grey are coefficients lower than 0.4, in blue are those greater than 0.7. Significant at the p value: *<0.05, **<0.01, ***<0.001. PA; Phytic Acid; GYD; Grain Yield.

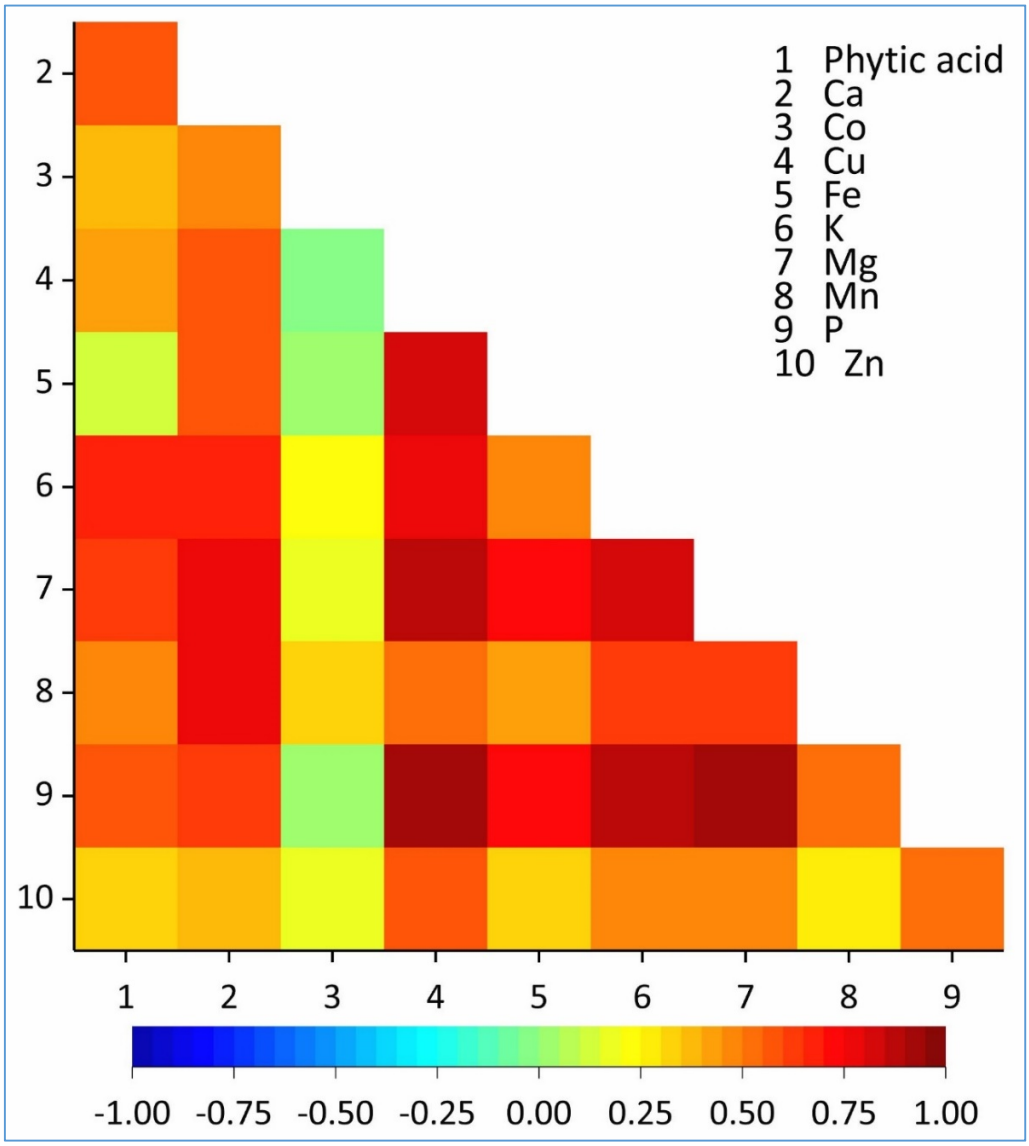



Figure 2-28 Relationship of wheat grain phytic acid concentration (mg per plant) with other grain mineral elements (mg $kg^{-1}$) in a hydroponic system with four levels of phosphorus. Data corresponds to three years. Colour corresponds to the strength and direction of the correlation from strongly negative (dark blue) to strongly positive (dark red). Ca, Cu, Fe, K, Mg, Mn, P, Zn (n=68), Co (n=52), Phytic acid (n=72).

#### *2.4.4.3 Phytic acid to mineral ratios*

Phytic acid millimolar ratios were calculated dividing the millimoles of PA by the millimoles of each of the mineral elements. The PA ratios of three important

minerals (Ca, Fe and Zn) were analysed using an unbalanced ANOVA blocking by set to assess the effect of the phosphorus treatments. All the ratios were significantly affected by the P treatment, interestingly the set played a major part on the variation.

In PA:Ca ratios, the unbalanced ANOVA was blocked by set; both the treatments were significant ($p<0.001$, LSD 1 %=0.2720), however, the set accounted for a larger percentage of the variation (34 %) compared to the treatments (22 %). Set Two had a mean significantly lower (1.333) than One and Three (1.87 and 1.83, respectively). Mean ratios for each treatment were 1.4, 1.7, 1.6 and 1.9, T1-4 respectively. We described previously that in Set Two instead of reducing the amount of $NH_4NO_3$, we reduced mistakenly the amount of $Ca(NO_3)_2$ (this was done at day 100 in order to stop the plants from producing more tillers. 44 days after this nitrogen reduction the plants were harvested Table 9-2. There were no evident consequences on plant growth and because the plants were in the last stages of maturation is very unlikely it could have affected grain composition. Calcium concentration was significantly different among the sets (513.1, 572.6 and 456.6 mg $kg^{-1}$, sets One, Two and Three respectively). However, set Two, had actually the highest concentration of Ca. Therefore, had the plants been deprived of Ca, a decrease in Ca should have been seen instead of an increase.

For PA:Fe ratios, same analysis was used, the treatments were significant ($p=0.026$, LSD 5 %= 4.001). The set accounted for a larger percentage of the variation (32 %) compared to the treatments (9 %). Set One had a mean

significantly lower (16.5) than sets Two and Three (22.9 and 27.40, respectively). In terms of treatments treatment 1 had the lowest mean value, 19.9, followed by T4 with 22.6 while T2 and T3 had 25.0 and 25.1 respectively.

In PA:Zn ratios, the treatments were significant ($p<0.001$, LSD 1 %= 12.94). The set accounted for a larger percentage of the variation (36 %) compared to the treatments (26 %). Set One had a mean significantly lower (30.8) than sets Two and Three (65.5 and 53.2, respectively). Mean values for each treatment were 38.9, 71.1, 48.7 and 53.2 in T1-4 respectively.

Lastly, for PAxCa:Zn ratios, the treatments were also significantly different ($p<0.001$, LSD 1 %= 156.2), were significantly affected by the phosphorus treatments. The set accounted for a larger percentage of the variation (49 %) compared to the treatments (17 %). The mean values were significantly different from each of the sets: 397.7, 900.2 and 600.5, for set One, Two and Three respectively. Mean values for each treatment were 459.5, 776.2, 679 and 767.7 in T1-4 respectively. Figure 2-29 shows the mean values for each of the treatments and mineral elements. All the values calculated were over the critical values which mean that the consumption of these grains by humans or animals will likely prejudice the bioavailability of these minerals. The critical value have been reported as: PA:Ca 0.24, PA:Fe 1, PA:Zn 15 and PAxCa:Zn 200.

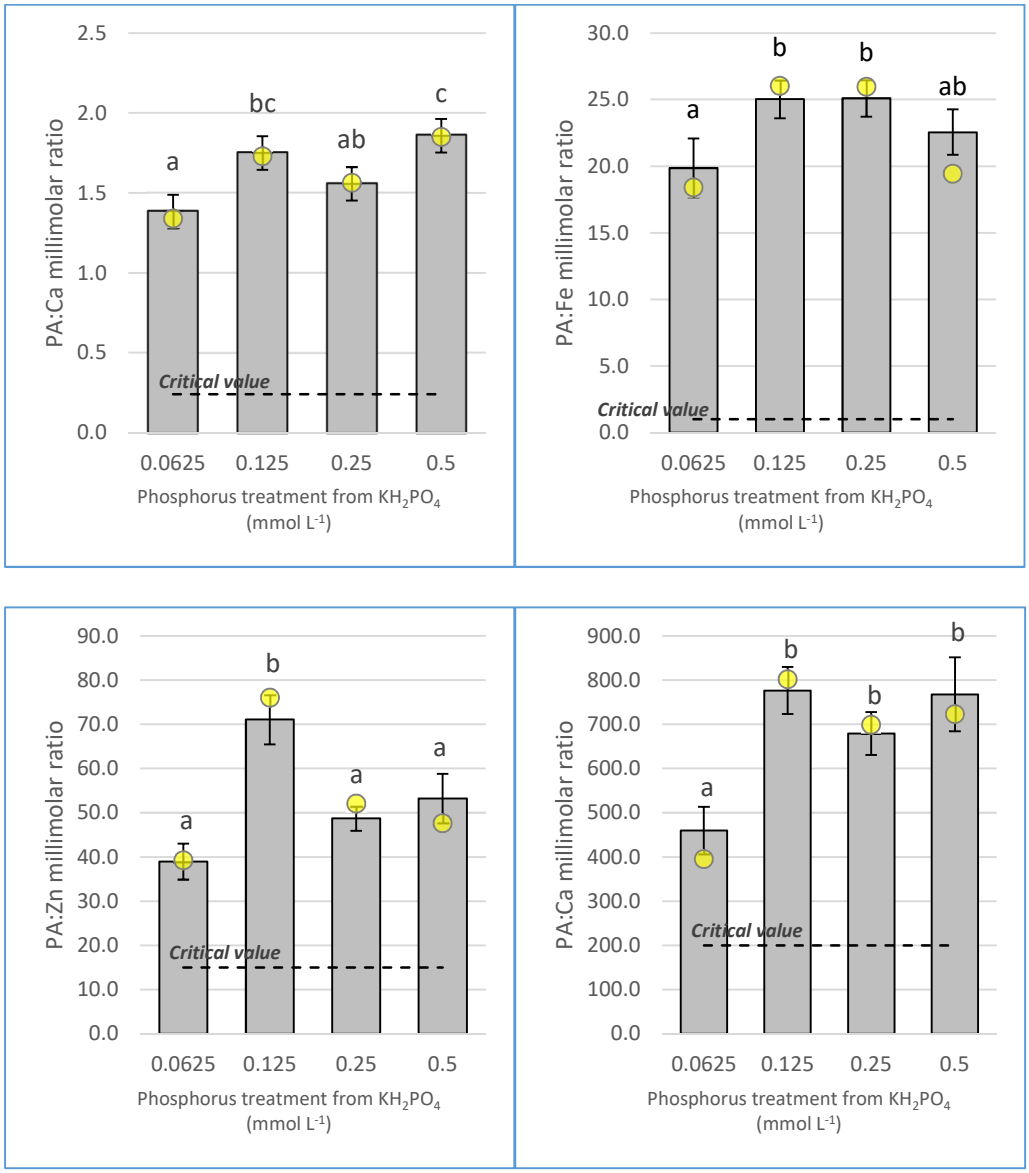



Figure 2-29 Phytic acid millimolar ratios in wheat grain as affected by different levels of phosphorus treatments in a hydroponic system. Means followed by a common letter are nor significantly different at the p=0.05 (PA:Fe) and p=0.01 (PA:Ca, PA:Zn, PAxCa:Zn). Unbalanced ANOVA and Fisher's unprotected LSD. Bars are means ± SE bars; yellow dots represent the median. Dotted lines are the critical values for each ratio. T1=0.0625 mmol $L^{-1}$, n=17; T2=0.125 mmol $L^{-1}$, n=16; T3=0.25 mmol $L^{-1}$, n=16; T4=0.5 mmol $L^{-1}$ n=19. Phosphorus from $KH_2PO_4$.

### 2.4.5 NFT grain zinc experiment

In total, 69 samples of wholegrain were analysed in three sets. The phytic acid concentration ranged between 4529 mg $kg^{-1}$ and 18085 mg $kg^{-1}$. A global mean of 12791 ± 3237 mg $kg^{-1}$ (mean ± SD). Medians for each year were: 8214, 13852 and 15922 mg $kg^{-1}$ (mean ± SD), for set One, Two and Three respectively.

The mean values by set were: 9196 ± 2282, 13845 ± 856.9 and 15464 ± 1784 mg $kg^{-1}$ for year 1, 2 and 3 respectively, (mean ± SD).

Unbalanced ANOVA was used to analyse this data, the results showed that the bench had no significant effect, unlike the set which was highly significant ($p<0.001$), and thus it was used as blocking factor. Table 2-14 shows a summary of phytic acid means by set and bench. Residuals were normal, Shapiro-Wilk test for Normality; Test statistic W=0.9886, p=0.786.

Table 2-14 Phytic acid mean, median and SD by set and bench of grain samples in an NFT zinc experiment. Data are in mg $kg^{-1}$.

| | | Bench A | | | | Bench B | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Set** | **Zn tx level** | **n** | **Mean** | **SD** | **Median** | **n** | **Mean** | **SD** | **Median** |
| 1 | 1 | 3 | 8003 | 310 | 8087 | 3 | 11741 | 1271 | 11206 |
| 1 | 2 | 3 | 7826 | 634 | 8071 | 3 | 12231 | 912 | 12419 |
| 1 | 3 | 3 | 7356 | 178 | 7337 | 3 | 9072 | 3935 | 11250 |
| 1 | 4 | 3 | 7545 | 550 | 7349 | 3 | 9790 | 1702 | 10592 |
| 2 | 1 | 3 | 13330 | 477 | 13222 | 3 | 14256 | 932 | 14769 |
| 2 | 2 | 3 | 14766 | 665 | 14656 | 2 | 12819 | 839 | 12819 |
| 2 | 3 | 3 | 13525 | 552 | 13680 | 3 | 14278 | 829 | 13946 |
| 2 | 4 | 2 | 14331 | 376 | 14331 | 2 | 12990 | 146 | 12990 |
| 3 | 1 | 3 | 16205 | 243 | 16193 | 3 | 16795 | 608 | 17028 |
| 3 | 2 | 3 | 17185 | 550 | 17187 | 3 | 13280 | 1071 | 13610 |
| 3 | 3 | 3 | 13966 | 1439 | 13933 | 3 | 15736 | 2146 | 15245 |
| 3 | 4 | 3 | 16193 | 1771 | 16542 | 3 | 14350 | 1921 | 14981 |

tx, treatment; SD, standard deviation; NFT, Nutrient Film Technique; n, number of values.

Zinc treatments had no significant effect ($p=0.252$) on the amount of phytic acid in wheat grain samples, mean values for each treatment were 13388 ± 3092, 13029 ± 3074, 12322 ± 3523 and 12392 ± 3407 (mean ± SD), treatment 1 to 4 respectively, Figure 2-30 top chart. However, there were significant differences among each of the sets ($p<0.001$), the set accounted for 71 % of the variance. Set One had the lowest PA concentration (9196 mg $kg^{-1}$) followed by the Set Two with 13845 mg $kg^{-1}$ and lastly, Set Three had the highest mean value, 15464 mg $kg^{-1}$ (means ± SD).

In terms of Phytic acid-P/total P %, we found 62.7 %, 62.9 %, 58.5 % and 58.5 % for treatments 1, 2, 3 and 4 respectively. For this calculation, the phytic acid-P which is determined by the Megazyme Kit is compared to the total P which is obtained by ICP-MS and then expressed as a percentage, Figure 2-30 bottom chart.

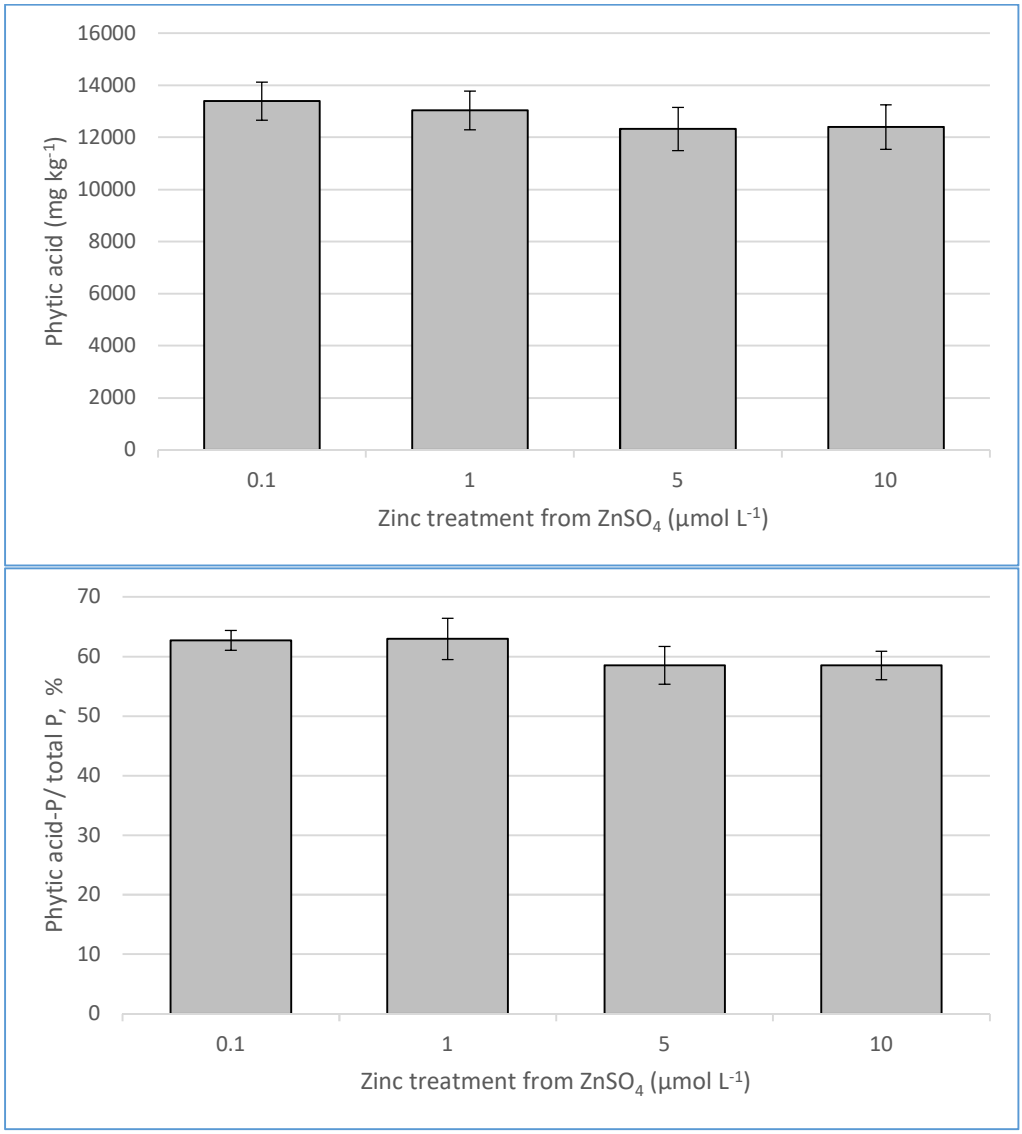



Figure 2-30 Phytic acid concentration in grain samples by treatment applied in the hydroponic system. Three-year data. Top chart, mean values ± standard error bars (SE). T1 n=18, T2 n=17, T3 n=18, T4 n=16. Bottom chart. Phytic acid-P/total P %. T1=0.1 µmol L-1, T2=1.0 µmol L-1, T3=5.0 µmol L-1, T4=10.0 µmol L-1 ZnSO4. Zinc treatment concentrations are expressed as micromole per litre (µmol $L^{-1}$). Treatment 1 and 2 n=17, treatment 3 n=18, treatment 4 n=16.

Similarly, zinc treatments did not have an effect on the grain yield (g per plant), p=0.658, or in the number of ears per plant, p=0.759. Mean values of grain yield

were 18.21 ± 7.019, 20.47 ± 8.146, 21.47 ± 8.669 and 19.50 ± 8.246 gram per plant for treatments 1-4 respectively, Figure 2-31.

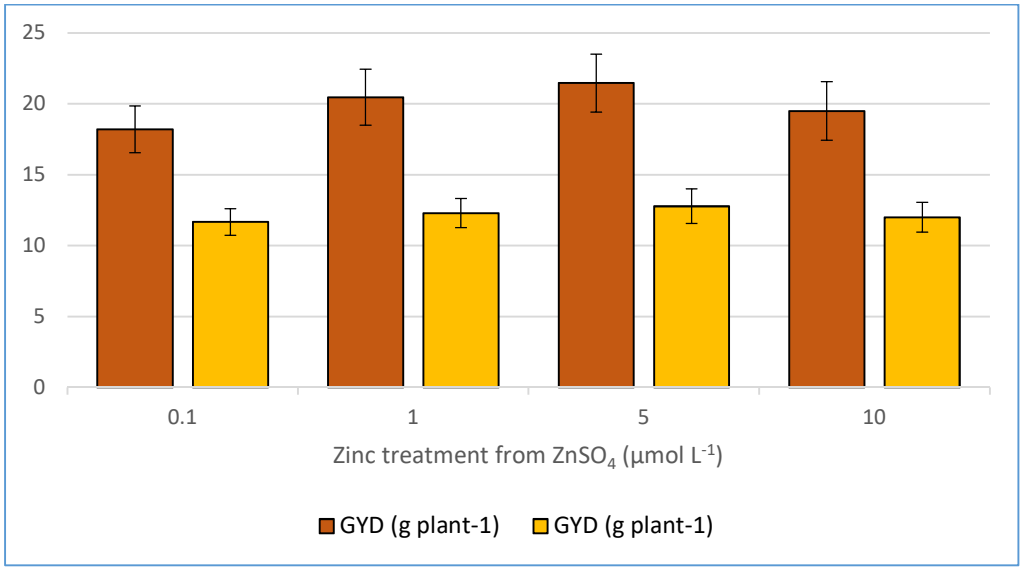



Figure 2-31 Grain yield (GYD, g plant$^{-1}$) and number of ears per plant of wheat grown in a hydroponic system with four levels of zinc. Data corresponds to three years. X-axis are zinc treatments, T1=0.1 µmol L$^{-1}$, T2=1.0 µmol L$^{-1}$, T3=5.0 µmol L$^{-1}$, T4=10.0 µmol L$^{-1}$ $ZnSO_4$. T1-T2 n=17, T3 n=18, T4 n=16.

The treatments did have a highly significant effect on the zinc concentrations in grain samples ($p<0.001$, LSD 1 %= 10.99). Fisher's unprotected LSD test showed that T1 and T2 had significantly lower Zn concentrations (17.8 and 18.2 mg kg$^{-1}$, respectively), compared to T3 (38.8 mg kg$^{-1}$) and T4 which had the highest Zn concentration (58.2 mg kg$^{-1}$), Figure 2-32.

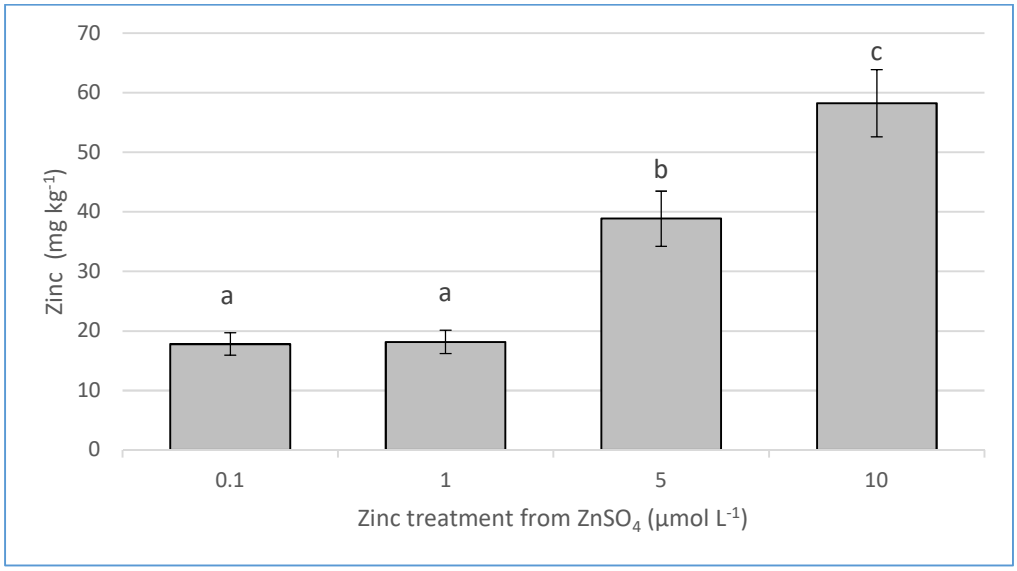



Figure 2-32 Zinc concentration (mg $kg^{-1}$) in grain samples as affected by zinc treatment applied in the hydroponic system. Mean values ± standard error bars (SE). Bars with the same letter are not significantly different at the p=0.01 level (Unbalanced ANOVA and Fisher's unprotected LSD test). X-axis shows the Zn concentration expressed in µmol $L^{-1}$. T1=0.1 µmol $L^{-1}$, T2=1.0 µmol $L^{-1}$, T3=5.0 µmol $L^{-1}$, T4=10.0 µmol $L^{-1}$ $ZnSO_4$. T1-T2 n=17, T3 n=18, T4 n=16.

### *2.4.5.1 Phytic acid and mineral interactions*

A correlation analysis was carried out including all the samples. There was a positive significant relationship between PA and P (r=0.640, p<0.001) and PA and Cu (r=0.299, p=0.046). PA and Mn (r= -0.331, p<0.026) also had a significant but rather weak negative relationship. PA had no significant relationship with either calcium (p=0.178), Fe (p=0.056) or Zn (p=0.344). Calcium had a strong positive relationship with Mg (r=0.860, p<0.001) and Mn (r=0.861, p<0.001). Magnesium and Mn also had a strong relationship (r=0.901, p<0.001). For zinc, although weak, the strongest association found was with Fe (r=0.396, p=0.007), Figure 2-33.

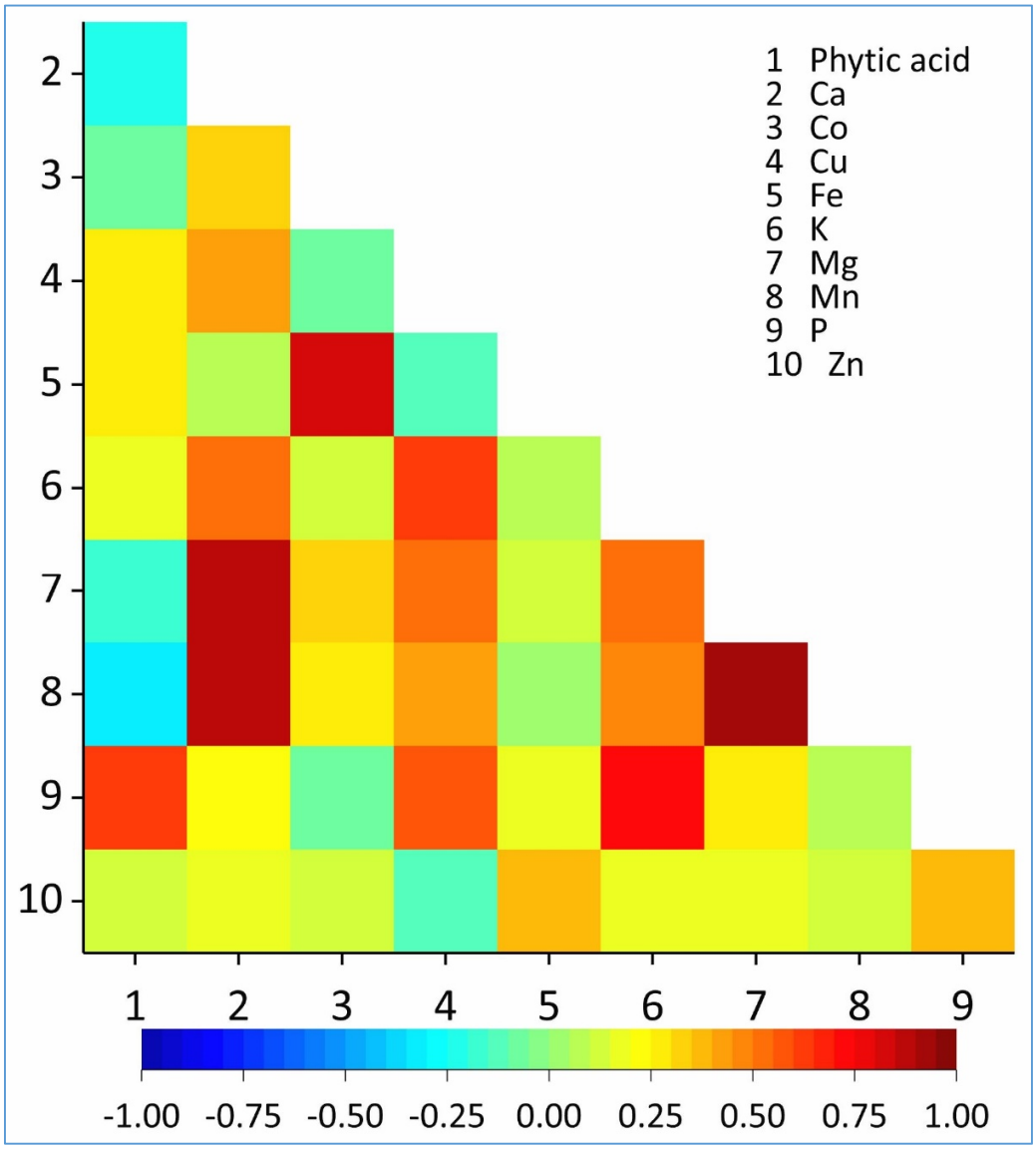



Figure 2-33 Relationship of wheat wholegrain Phytic acid concentration (mg $kg^{-1}$) with other grain mineral elements in a hydroponic system with four levels of zinc. Data corresponds to three years. Colour corresponds to the strength and direction of the correlation from strongly negative (dark blue) to strongly positive (dark red). Ca, Fe, K, Mg, Mn, P, Zn (n=68), Co, Cu (n=45), Phytic acid (n=69).

#### *2.4.5.2 Effect of treatments on mineral concentrations*

Mineral concentrations were analysed using an unbalanced ANOVA blocking by set and Fisher's LSD as a post hoc test to understand the effect of zinc treatments in wheat grain. Out of nine minerals analysed, only two were significantly affected by the treatments: copper and zinc. Copper concentration was affected significantly by zinc treatments ($p=0.006$, LSD 1 %=0.8548), the highest mean concentration was observed in T1 (9.73 ± 0.636 mg $kg^{-1}$), the next highest was in T4 (8.78 ± 0.845 mg $kg^{-1}$). Treatment 4 was no different from either T1 or T2-3 (8.70 ± 0.801, 8.78 ± 0.813 respectively). Zinc concentrations were influenced significantly ($p<0.001$) as it was mentioned before, Figure 2-34.

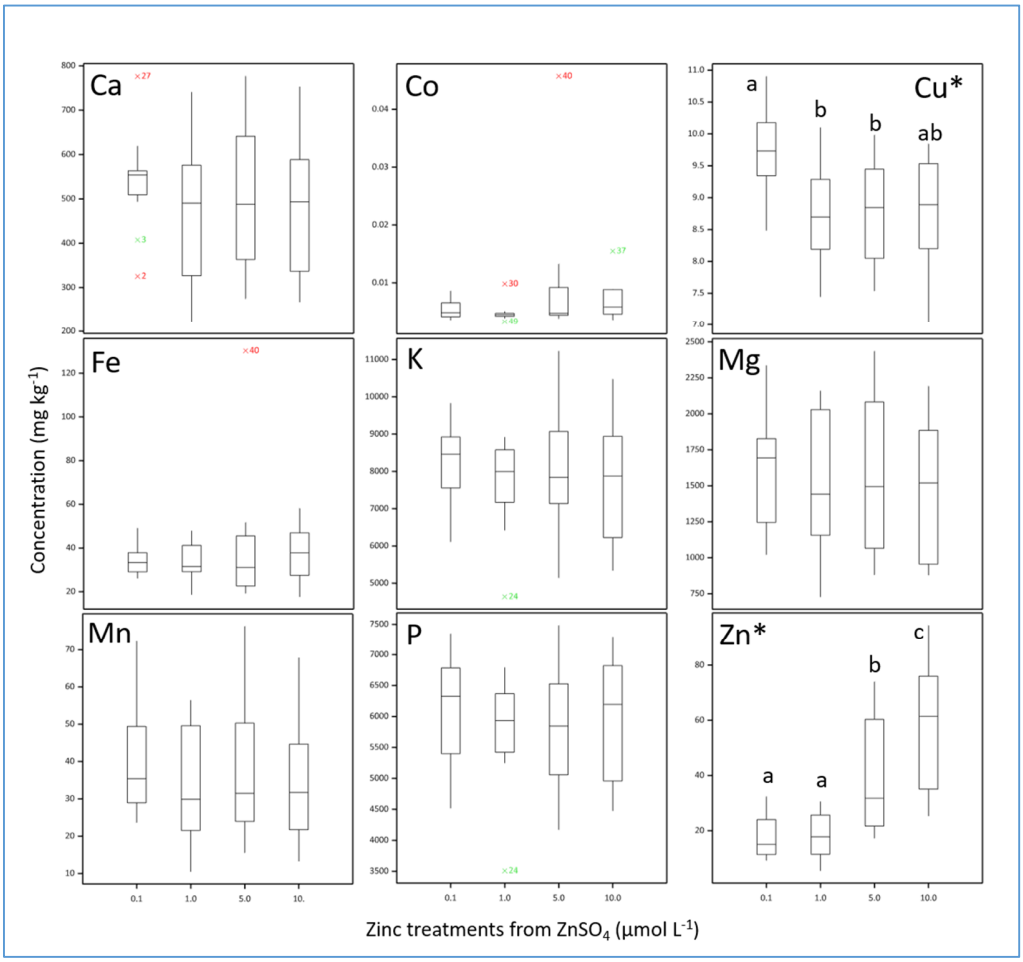



Figure 2-34 Mineral concentrations in wheat grain (mg $kg^{-1}$) grown in a hydroponic system as affected by different levels of zinc supply. X-axis shows zinc treatments from $ZnSO_4$ (µmol $L^{-1}$). Data corresponds to three years. Bars with the same letter are not significantly different at the p=0.01 level (Unbalanced ANOVA and Fisher's unprotected LSD test). Total n for each element: Ca, Cu, K, Mg, Mn, P, Zn n=68, Co, Cu n=45. T1=0.1 µmol $L^{-1}$, T2=1.0 µmol $L^{-1}$, T3=5.0 µmol $L^{-1}$, T4=10.0 µmol $L^{-1}$ of $ZnSO_4$. Schematic boxplot shows the interquartile range with the median drawn; whiskers are 1.5 times the interquartile range beyond the quartiles, or the maximum value if that is smaller. Green crosses are outliers.

### *2.4.5.3 Phytic acid to zinc molar ratio*

In addition, phytic acid millimolar ratios were calculated dividing the millimoles of

PA by the millimoles of each of the mineral elements. Analysis by unbalanced

ANOVA blocked by set and Fisher's unprotected LSD test, showed that only the PA:Zn ratios were affected significantly by the treatments ($p<0.001$, LSD 1 %=10.99).Ratios decreased with the increasing concentrations of Zn in the nutrient solution. Mean values were 86.01, 83.24, 35.89 and 22.58 for T1 to T4 respectively, Figure 2-35.

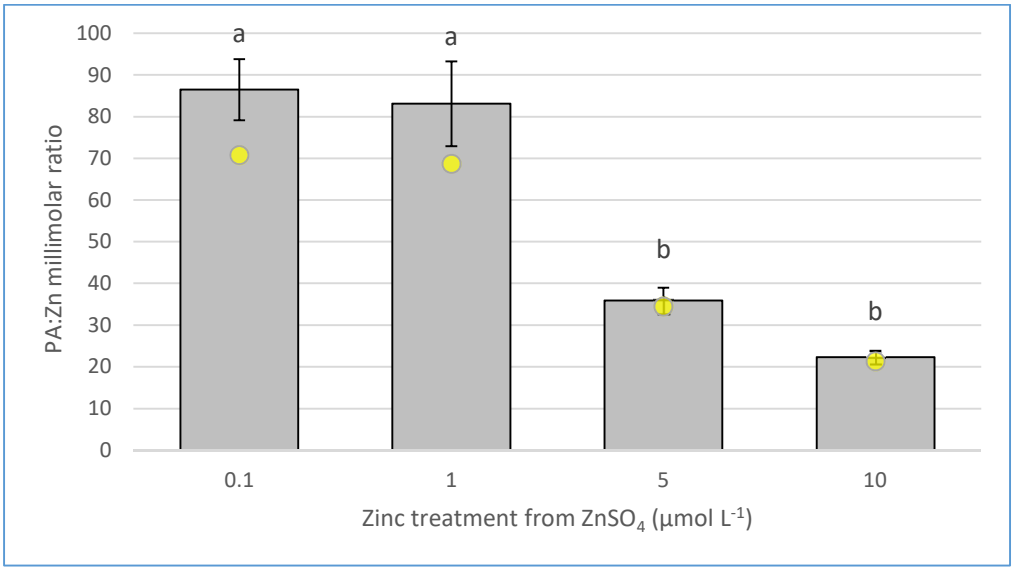



Figure 2-35 Phytic acid to zinc (PA:Zn) millimolar ratios as affected by zinc treatments in wheat grain grown in a hydroponic system. Bars with the same letter are not significantly different at the p=0.01 level (Unbalanced ANOVA and Fisher's unprotected LSD test). Bottom numbers are treatment concentration levels from $ZnSO_4$: T1 0.1 µmol $L^{-1}$ n=17, T2 1.0 µmol $L^{-1}$ n=17, T3 5.0 µmol $L^{-1}$ n=18, T4 10.0 µmol $L^{-1}$ n=16.

### 2.4.6 Relationship between leaf and grain phytic acid and minerals

To explore any associations of phytic acid and mineral concentrations between the leaf and the grain and establish the possibility of using leaf as a proxy for grain PA concentration, we ran a correlation analysis, separately for phosphorus and

zinc experiments and using all the samples (i.e., no grouping by treatments). The results for phosphorus experiment are described first and are followed by the results of zinc experiment.

For phosphorus experiment, magnesium and phosphorus showed the strongest relationship (Mg r= 0.881, p<0.001; P r=0.829, p<0.001), followed by Ca (r= 0.558, p<0.001) and Zn (r= 0.482, p<0.001) whereas Mn (r= 0.373, p=0.003) and PA (r= 0.370, p=0.001) displayed a rather weak relationship. Potassium on the other hand, was the only element that exhibited a negative relationship (r= -0.435, p=0.001).

Finally, there was a high association of P concentrations in leaf with the PA concentration found in grain (r= 0.638, p<0.001). There was no relationship observed on Co, Cu, Fe and Phytic acid-P/ total P (%). Relationship between the leaf PA and grain minerals were not quite strong, the strongest of them was the PA in leaf with P in grain ($r^2$= 0.546, p<0.001), followed by PA in leaf and Mg in grain ($r^2$= 0.475, p<0.001). There was a very weak negative relationship but not significant, between the phosphorus concentration in leaf and the phytic acid-P/total P (%) in grain (r= -0.009, p=0.943). Schematic representations can be found in Figure 2-36 and the whole set of leaf-grain mineral combinations can be found in Figure 2-37.

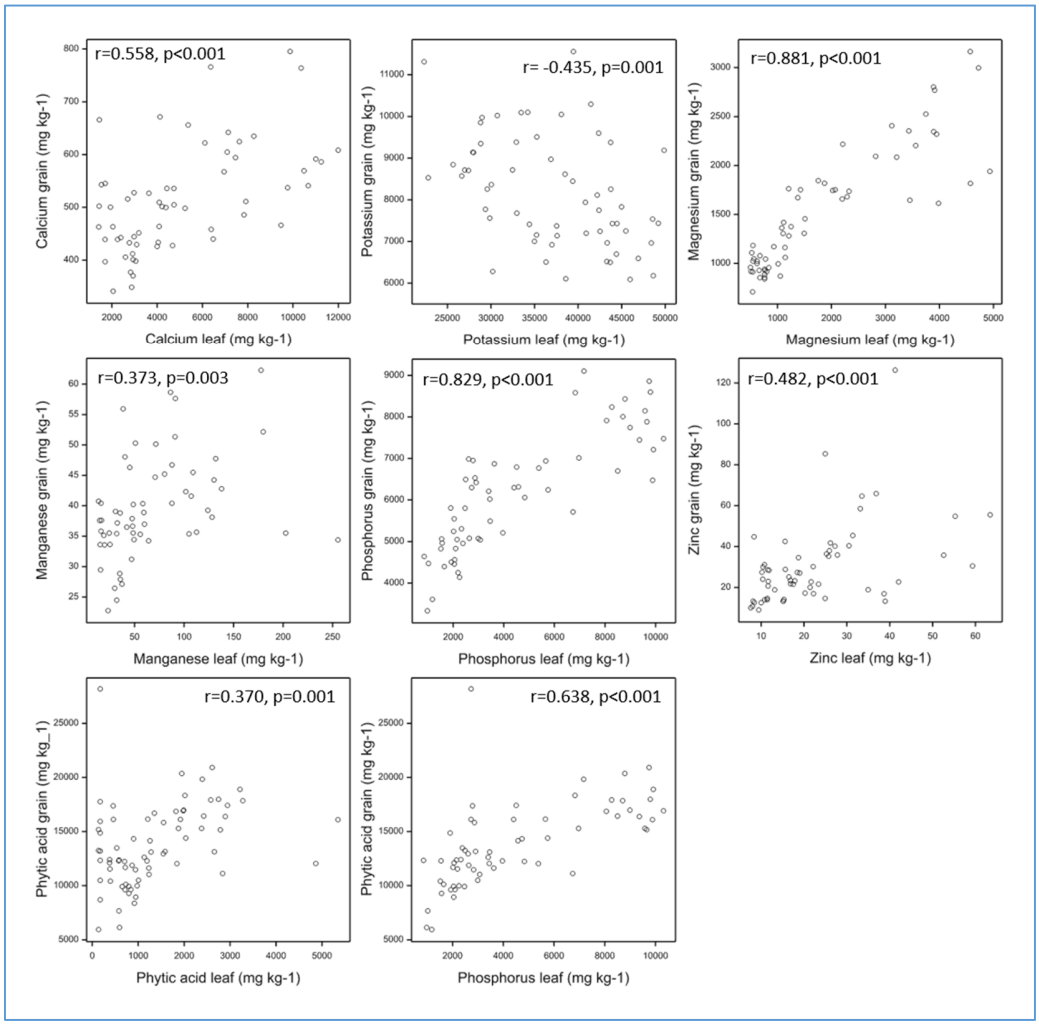



Figure 2-36 Relationship between mineral and phytic acid concentrations in wheat leaf and wholegrain grown in hydroponic system with controlled phosphorus supplementation. Concentrations are in mg $kg^{-1}$. Scatter plot shows three year and four treatment data for each element, r and p value.

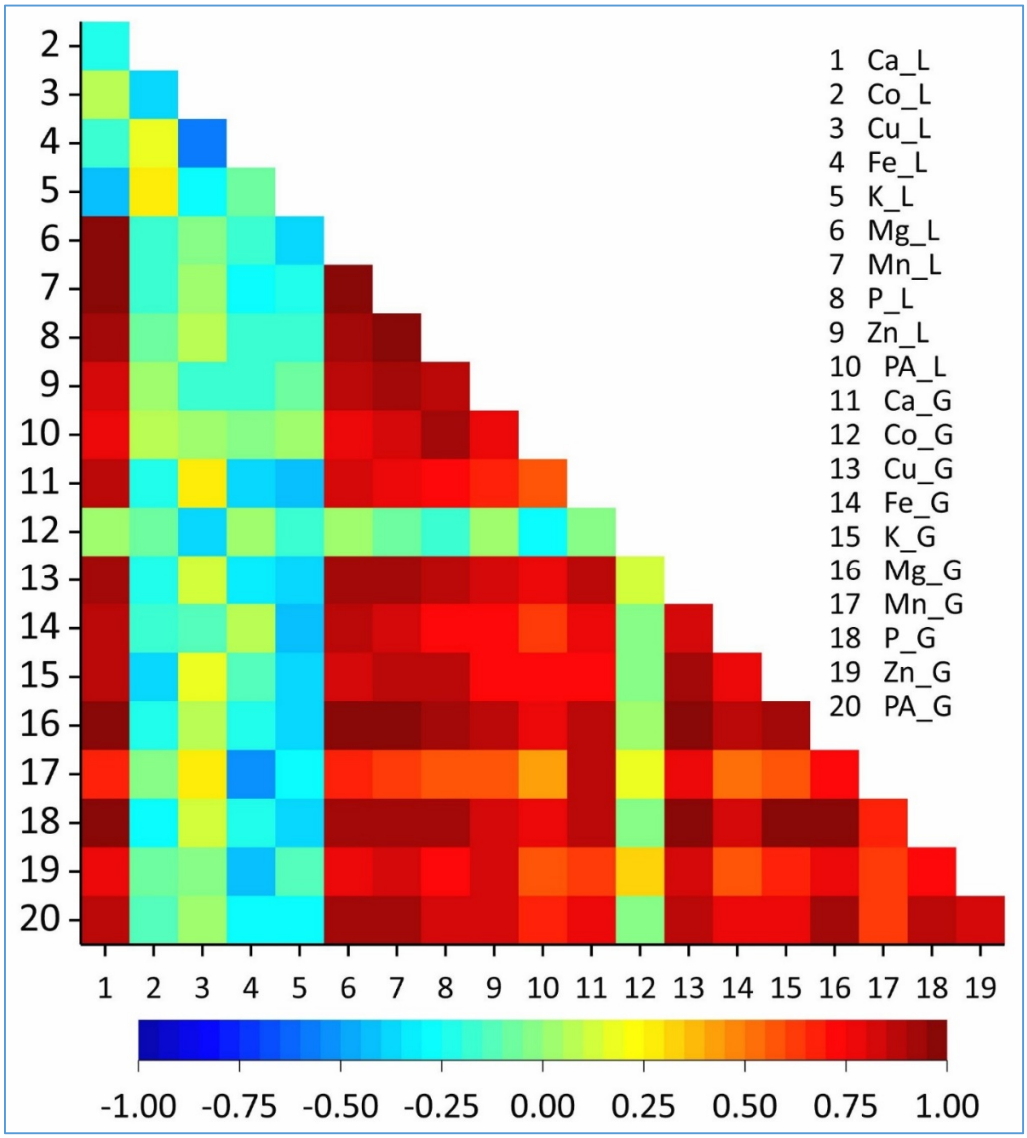



Figure 2-37 Relationships between leaf-grain minerals and phytic acid (PA) concentrations. Wheat Paragon grown in a hydroponic system during three years and under four different levels of phosphorus. Concentrations are in mg $kg^{-1}$. Colour corresponds to the strength and direction of the correlation from strongly negative (dark blue) to strongly positive (dark red). L=leaf sample, G=grain sample.

Additionally, a simple linear regression was calculated to predict the mineral concentrations in grain based on their concentrations in leaf and to predict phytic acid concentrations in grain from the phosphorus concentration in leaf (last

equation shown in Table 2-15). It is important to note that the regressions were calculated without taking into account the treatments. All the linear equations (except Mn, p=0.003) were highly significant (p<0.001). The equation for Mg was the one explaining the highest percentage of variability (77.5 %) followed by P (68.1 %) and P leaf/PA grain (39.7 %). The equations for zinc and phytic acid, should be taken with care due to GenStat warning of non-random residuals. A summary of the equations can be found in Table 2-15.

Table 2-15 Linear regression equations for minerals in wheat grown in hydroponic system with four levels of phosphorus.

| | **Equation** | **p value** | **SE** | **% Variance** |
|---|---|---|---|---|
| Calcium$^{+}$ | y= 0.01897x + 418 | <0.001 | 85.2 | 30 |
| Potassium$^{+}$ | y= -0.0786x + 11113 | <0.001 | 1205 | 17.5 |
| Magnesium$^{+}$ | y= 0.4031x + 784.4 | <0.001 | 295 | 77.5 |
| Manganese$^{+}$ | y= 0.0600x + 35.35 | 0.003 | 7.74 | 12.4 |
| Phosphorus$^{+}$ | y= 0.3873x + 4390 | <0.001 | 796 | 68.1 |
| Zinc$^{+}$ | y= 0.726x + 13.86 | <0.001 | 17.7 | 21.9 |
| Phytic acid$^{+}$ | y= 1.270x + 12016 | <0.001 | 3537 | 12.5 |
| P leaf/PA grain$^{\Delta}$ | y= 0.829x + 10070 | <0.001 | 2988 | 39.7 |

Note: treatments were not considered for this analysis. SE, standard error of the observations; % Variance, % of the variance explained by the linear model. $^{+}$ Equation to estimate the concentration of the mineral in grain from its concentration in leaf; $^{\Delta}$ Equation to estimate phytic acid (PA) in grain from the P concentration in leaf.

In the case of the zinc experiment, Ca, Co, Cu and K had a significant relationship between the concentrations in leaf and those in grain, but the associations were rather weak. Ca, r= -0.387, p=0.002; Co, r= -0.360, p=0.019; Cu, r= 0.428, p=0.003 and K, r= -0.316, p=0.011. Only zinc had a rather high association r= 0.659, p<0.001, Figure 2-38. For the rest of the minerals no significant association was found. Moreover, phosphorus and or phytic acid concentrations were not linked to zinc concentrations and also we found that contrary to phosphorus experiment, phosphorus concentration in leaf had no significant relationship with PA in grain (r= 0.007, p=0.954). Figure 2-39, illustrates other leaf-grain mineral combinations.

Similarly, the regression analysis was carried out for the five elements just mentioned, all the equations were significant but the percentage of variance they accounted for was low, 13.6, 10.8, 16.4 and 8.5 % for Ca, Co, Cu and K respectively. Zinc regression on the other hand, was highly significant (p<0.001) and accounted for 42.5 % of the variation found, y=1.168x + 4.79, SE=17.5. Just as it was stated in previous paragraphs, this analysis did not take into account the treatments.

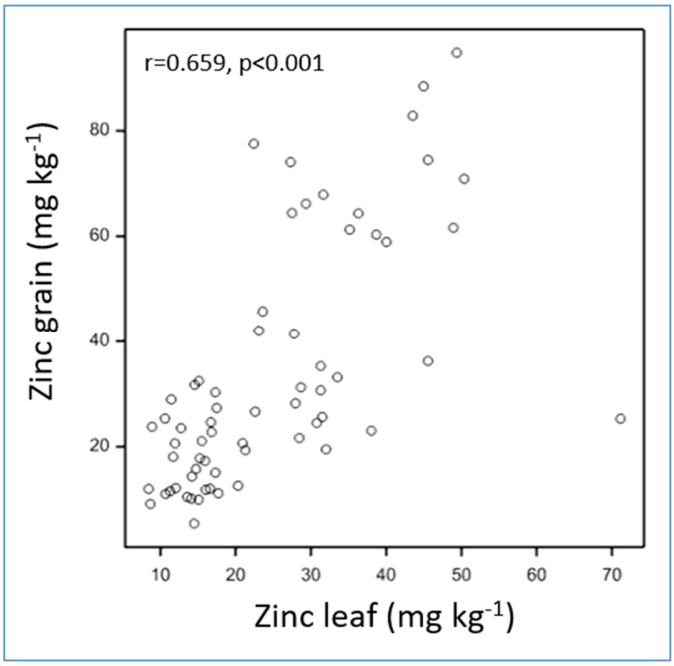



Figure 2-38 Relationship between zinc concentrations in wheat leaf and grain grown in hydroponic system with controlled zinc supplementation. Concentrations are in mg $kg^{-1}$. Scatter plot shows three year and four treatment data for each element, r and p-value.

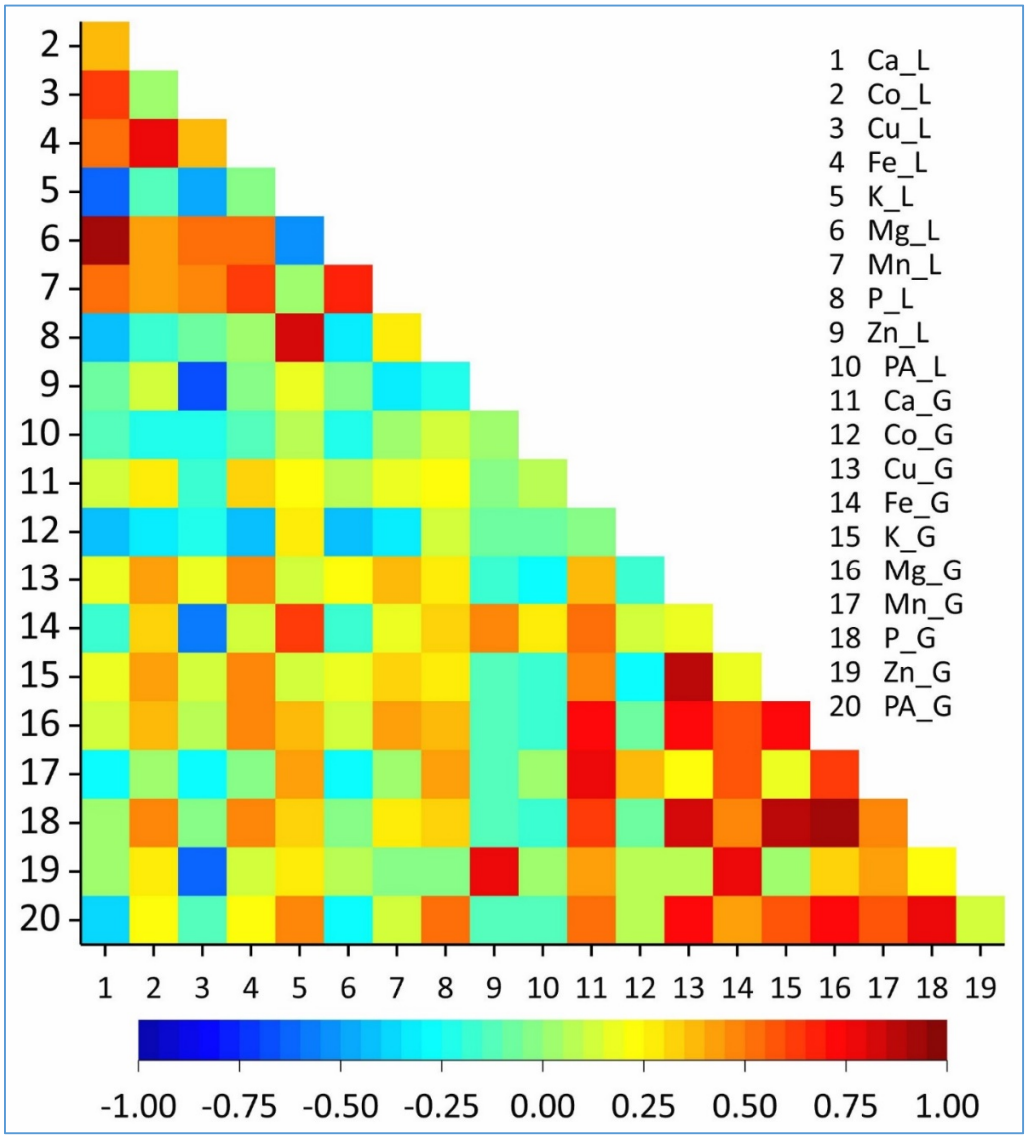



Figure 2-39 Relationship between leaf and wholegrain mineral and phytic acid (PA) concentrations. Wheat Paragon grown in a hydroponic system during three years and under four different levels of zinc. Concentrations are in mg $kg^{-1}$. Colour corresponds to the strength and direction of the correlation from strongly negative (dark blue) to strongly positive (dark red). L=leaf sample, G=grain sample.

## 2.5 DISCUSSION AND CONCLUSIONS

### 2.5.1 Phytic acid concentrations in leaf and wholegrain samples

The phytic acid (PA) range in wheat leaves in P experiment fertilization was 376.72 to 5348 mg $kg^{-1}$. Leaf samples under controlled Zn fertilization had similar concentrations of PA varied in a range of 603.82 to 5288 mg $kg^{-1}$ and a global mean of 2054 ± 944.5 mg $kg^{-1}$ (mean ± SD). It is been established that PA levels in leaf are generally low (DeTurk et al., 1933; Oberleas, 1973), however, in some plants such as Bromeliads, P is stored in leaves as PA (<20 % of the total P) as a response to poor nutrient environments and limited P supply. The plant would do what is called luxury consumption when there is a surplus of P in the media and store it to sustain later growth. Generally high PA in leaves indicate an excess of P whereas the absence is indicator of insufficient P (Wanek & Zotz, 2011; Winkler & Zotz, 2009). The literature contains few reports on PA concentrations in leaves mainly because it is considered negligible and studies available would focus on edible leafy greens used for human consumption.

Our leaf PA values were higher than those reported by Ravindran (1994) in spinach and sweet potato (700 mg $kg^{-1}$) and in underutilized green leafy vegetables (fresh weight) 9.2-130.6 mg $kg^{-1}$ (Gupta et al., 2005). But quite similar to those reported by Zhao (2007) in *Brassica* leaves (0-3400 mg $kg^{-1}$), Ravindran (1994) in Cassava leaf meal (phytic acid-P=9.5 %; PA=1400 mg $kg^{-1}$) and Raboy & Dickinson (1984) in soybean leaf, reported 1000-6200 mg $kg^{-1}$ of phytic acid. Alkarawi & Zotz (2014a, 2014b) in a revision of different green leafy species found an average of 2500 mg $kg^{-1}$ with a range between 300 – 29000 mg $kg^{-1}$.

Grain samples in our P experiment had a mean of 13737 mg $kg^{-1}$ with a range of 5948 to 27178 mg $kg^{-1}$. On the Zn experiment, the values were quite similar; the global mean was 12791 ± 3237 mg $kg^{-1}$, with a much lower range, 4529 to 18085 mg $kg^{-1}$. Shitre (2015) reported an average of 49700 mg $kg^{-1}$ in diverse breeding lines of wheat, which is higher than what we obtained. Liu (2006) in Chinese wheat reported PA content of 7800 mg $kg^{-1}$ with a range of 5160 to 9870 mg $kg^{-1}$, which is within the range of our samples.

### 2.5.2 Phytic acid-P as a percentage of total P (Phytic acid-P/total P %) is not affected by the P or Zn supply.

Alkarawi & Zotz (2014a, 2014b) reported in leaf samples that phytic acid-P accounted for 1-27 % of the total P. Our leaf samples were within this range, the percentage of phytic acid-P relative to total P was not significantly different among the treatments, and for example, we observed 7.7-11.7 % on the P experiment whereas on the zinc experiment, we observed 7.7 – 10.3 %, still within the range of previous reports. Ravindran (1994) for example, found phytic acid-P was 4.5 % of total P in spinach and 6.1 % on sweet potato leaves.

Likewise, in wholegrain samples, neither of the treatments had any effect, phytic acid-P as a percentage of total P was relatively constant, our highest percentage in the P experiment was found in T2 with 66.62 % and the lowest was in T3 60.72 %. Meanwhile in our Zn experiment the percentages observed were 62.7 %, 62.9 %, 58.5 % and 58.5 % for each treatment. It is well known the majority of P in grains is found as PA, for example Lott (Lott et al., 2000) mentioned that in normal,

non-mutant seeds of cultivated species, phytic acid P typically represents greater than 65% of grain total P, and grain total P and phytic acid-P are highly and positively correlated. Likewise, Ravindran (1994) described in cereal grains, oilseeds and grain legumes, high levels of phytic acid, and phytate P constituted the major portion (60-82 %) of total P. The proportions that we found in our study agree with those in previous reports.

We did not find a significant relationship between the ratio of phytic acid-P and total P in leaves ($r^2$= -0.157, p=0.204); Alkarawi & Zotz in their studies with leaves (2014a, 2014b) found the proportion tended to decrease although not significantly. They state that the lack of a positive correlation, is puzzling because generally, not universally, what is observed is a positive association in mature seeds and fruits (Eeckhout & De Paepe, 1994). Zhao (2007) reported a correlation coefficient between phosphate and phytate levels, r= 0.52, p<0.01, in *Brassica* leaves and r= 0.44, p<0.01 in seeds.

### 2.5.3 Phosphorus treatments affect PA concentrations in wheat leaves and wholegrain whereas zinc treatments affect PA in leaves but not in the wholegrain.

In this study, the phosphorus treatments had significant effect over the PA concentration in wheat leaves, the amount of PA increased 354.8 % in treatment 4, compared to treatment 1. In the experiment with Zn treatments, treatments also had an effect on the PA in leaves, we observed an increase of about 20 % from

T1 to T2, 3, and there were no differences between 2 and 3 and then the concentration increased about 17 % on T4.

For grain samples, we observed PA concentrations were around 27 % higher in treatment 2 compared to treatment 1. Both T2 and 3 were no different from each other and in treatment 4, the PA increased again about 24 %. On the contrary, zinc treatments did not have an effect over the PA concentrations, mean values for each treatment were 13388 ± 3092, 13029 ± 3074, 12322 ± 3523 and 12392 ± 3407 (mean ± SD treatment, treatments 1 to 4 respectively).

Zinc treatments did have an effect on the zinc concentrations in grain, from 17.8 and 18.16 mg $kg^{-1}$ on T1 and 2, which were no different from each other, Zn concentrations increased 113.7 % on T3, and a further 50 % increase in T4, compared to T3. As a summary, Zn concentrations increased 220.5 % from T2 (1.0 µmol $L^{-1}$) to T4 (10 µmol $L^{-1}$).

Although our experiment was not a factorial combination of P and Zn like that done by Raboy & Dickinson (1984) in soybean, we observed similar results. In their experiment the authors reported PA increased around 200 % with the increasing levels of nutrients (6300-19100 mg $kg^{-1}$) and seed Zn increased from 62-88 mg $kg^{-1}$ (42 % increase). The authors also stated that the highest nutrient Zn treatment tended to cause a decrease in seed phytic acid levels, at all P levels and the same tendency was reported by (Z. Wang et al., 2015) who reported PA concentrations in grain decreased from 9900 mg $kg^{-1}$ to 7200-9000 on the highest Zn treatments.

Yang (Yang et al., 2011) equally observed that P supply increased PA levels in grain, and that the addition of Zn further decreased the PA in grain. Therefore, PA in grain could be affected by Zn application rates; which we did not observe in our experiment.

Perhaps the fact that we tested both minerals independently and not as combinations as these authors did. Previous findings suggest the interaction of both P and Zn might be the cause of the phytic acid decrease. An explanation of the interactions could be that both minerals are controlled by polygenes, and QTLs for Zn control are close to those controlling phosphorus (R. Shi et al., 2008).

The standard solution for hydroponics in our laboratory was T2=1.0 µmol $L^{-1}$ $ZnSO_4.7H_2O$=65.38 µg $L^{-1}$ zinc. Raboy & Dickinson (1984) stated that nutrient Zn levels above that of the standard Hoagland solution (50 µg $L^{-1}$) resulted in an increase in seed Zn in the range of 60-70 mg $kg^{-1}$ to over 100 mg $kg^{-1}$, our largest increase was around 60 mg $kg^{-1}$ which is higher than that reported by Wang (2015) who described that Zn concentrations increased in the grain 60-140 % and 100-150 % in two different years with Zn application to soil.

### 2.5.4 PA mineral interactions in leaf and wholegrain; PA is strongly associated with P.

In leaf samples from the P experiment, PA had a strong relationship with Ca, Mg, Mn, P and Zn ($p<0.01$). Phosphorus was also significantly related to the concentrations of Ca, Mg, Mn and Zn ($p<0.001$). Interestingly, in the zinc

experiment no relationship was found between PA and any other mineral. Phosphorus had a positive strong association with K ($p<0.001$) and Zn had a negative strong relationship with Cu ($p<0.001$). Bawa & Yadav (1986) in green vegetables found significant relationships between P and Fe ($r=0.81$, $p=0.05$), P and Mg ($r=0.97$, $p=0.01$) concentrations whereas P and PA ($r= -0.26$) was not significant. The significant correlations observed here between P and other cations demonstrate their relationship during the uptake and transport in leafy vegetables.

In our grain samples on the P experiment, PA was related to Cu, Fe, K, Mg, P and Zn ($p<0.001$). PA and P concentrations were described by the linear equation: $y= 2.165x + 536$. For samples in the zinc experiment, PA and P had a positive relationship ($r=0.640$), PA and Cu association was rather weak ($r=0.299$), and a negative and rather weak relationship was observed for Mn ($r= -0.331$). On the other hand Ca had a strong positive relationship with Mg ($r=0.860$) and Mn ($r=0.861$). Zn was only associated weakly with Fe ($r=0.396$). Lolas (1976) had also reported PA concentrations were highly correlated with total P concentrations in wheat grain ($r=0.9682$), indicating that PA concentrations could be used to estimate the total phosphorus concentration. Erdal (2002) in their study discussed the possibility of reducing P concentrations by Zn application of soil, for example, they observed reductions of P from 3900 to 3500 mg $kg^{-1}$ and PA decreases of 10700 to 9100 mg $kg^{-1}$. Contrary to our results, Su (2018), reported a negative correlation of PA with Fe and Zn.

### 2.5.5 P and Zn treatments had a significant effect over the concentrations of other minerals.

In leaf samples, P treatments significantly affected the concentrations of Ca, Cu, Mg, Mn, P and Zn, showing an increasing tendency, K was the exception as its concentrations tended to decrease with the increasing P supply. Zinc treatments had a significant effect on the concentrations of Ca, Cu, Fe, K, Mg, Mn and Zn. Alkarawi & Zotz (2014b) reported increasing P concentrations in leaves of *P. oficinale* with increasing levels of P supply combined with nitrogen supply, but the proportion phytic acid-P/total P, % as a function of total P was unchanged in all treatment combinations. Our results agree with these findings, the proportions of phytic acid-P/total P (%) were not affected by the treatments.

Zinc concentrations in our wheat leaves increased 68.28 % in T3 and 156.13 % in T4 from 16.55 mg $kg^{-1}$ in T2. Generally, the Zn concentrations in plant leaf are between 15 and 100 mg $kg^{-1}$ (Zhao, 1996). The plant is considered to be deficient of Zn if the leaf Zn concentration is lower than 15–20 mg $kg^{-1}$ (dry weight, DW), (Tongke, 1996), according to this, our plants in T1 were Zn deficient (13.47 mg $kg^{-1}$) and far from Zn toxicity which is exhibited when Zn concentration is greater than 400-500 mg $kg^{-1}$ dry weight (Jin, 1996) as cited in (Z. Wang et al., 2015). Wang (2015) also reported that leaf Zn concentrations increased significantly with Zn application rates 3.4- to 5.8-fold and 6.8-fold compared to treatment zero, their values (23.0-156.8 mg $kg^{-1}$ and 37.5-295.6 mg $kg^{-1}$), were higher than ours.

In the experiment by Raboy & Dickinson (1984) P and Zn concentrations increased in soybean leaf in response to treatments, in our study Zn increased with either P and Zn treatments , whereas P increased only with P treatments. The authors also reported that their highest Zn treatment reduced leaf P, which was not observed in this study. A possible explanation for this could be that the authors tried a combination of treatments and not each nutrient individually as we did. The translocation of PA in grain is highly dependent on the root uptake of P and its translocation form leaves to the seeds, at the same time these two mechanisms (root uptake and shoot accumulation) are greatly affected by zinc deficiencies (Erdal et al., 2002).

In wholegrain samples, P treatments affected significantly the concentrations of eight minerals. Comparing T1 to T4 (highest), Ca had an increase of 21.5 %, Cu 66.8 %, Fe 37.7 %, K 35.3 %, Mg 111 %, Mn 18 %, P 72.4 % and Zn 30.4 %. Zinc treatments had a significant effect on the concentrations of Cu ($p=0.006$, LSD 1 %=0.8548) and Zn ($p<0.001$, LSD 1 %=10.99), which was described before. Our Zn concentrations (17.8-58.2 mg $kg^{-1}$) for example, were higher than those reported in field grown Chinese wheat varieties, 16.2-32.4 mg $kg^{-1}$ (Z. H. Liu et al., 2006) and in advanced breeding lines grown in India 17.2-28.9 mg $kg^{-1}$ (Shitre et al., 2015) but similar to those reported by Graham (1999) in *T. aestivum* in Mexico.

### 2.5.6 Phytate to mineral ratios were affected by treatments.

PA:Fe and PA:Zn ratios were calculated in leaf samples. We observed these had an influence from the P treatments. PA:Fe millimolar ratios increased 481 % in T4 compared to T1. PA:Zn ratios, we observed an increase of 215.6 % from T1 to T3 and interestingly in treatment 4 the levels dropped 28 % compared to those of T2. PA:Fe ratios calculated for our samples, were higher than the critical value of <1 whereas PA:Zn ratios were below the critical values of <15.

On our zinc experiment, the treatments also had an effect on the mineral ratios, for example, PA:Fe ratios increased with the concentrations of Zn supply, we observed an increase of 80 % when Zn concentrations were increased from 0.1 µmol $L^{-1}$ to 10 µmol $L^{-1}$ (T1 to T4), and again, all the values were above the critical value (<1). PA:Zn ratios were affected in a different way, there were no differences between 0.1 and 1.0 µmol $L^{-1}$ treatments whereas with 5 µmol $L^{-1}$ treatment, the ratio fell 44 % and with the 10 µmol $L^{-1}$, it decreased further 18.7 %. The two highest Zn treatments yielded PA:Zn ratios within the recommended value for good zinc bioavailability. Although wheat leaves are not consumed by non-ruminants, the report can give insight of the PA accumulation in other leafy crops.

Unfortunately, currently there is very little information about PA ratios in leaves, so it is difficult to compare results. However, the FAO & IZiNCG (2018), released a fantastic compilation of phytate data from the literature for raw and processed food. Most of the information available for vegetables is for fresh samples. Accessions of dried leaves include plants such as amaranth (PA= 780 mg $kg^{-1}$),

baobab (PA=2600 mg $kg^{-1}$; PA:Zn=7.15), bitterleaves (PA=34.4 mg $kg^{-1}$; PA:Fe=0.5; PA:Zn= 0.07), blacknightsade (PA=68.9 mg $kg^{-1}$; PA:Fe=0.14; PA:Zn=0.18), Jew's mallow (PA=345.4 mg $kg^{-1}$; PA:Fe=0.07; PA:Zn=1.07), moringa (PA=21574 mg $kg^{-1}$), pumpkin (PA=150 mg $kg^{-1}$) and sicklepod (PA=176.2 mg $kg^{-1}$; PA:Fe=0.02; PA:Zn=0.64). In the database, data is presented as mg 100 $g^{-1}$ of EP (edible portion) and expressed as fresh weight. Moreover, PA on each accession had been determined by different methods, and none of them was Megazyme kit.

Amaranth, baobab and Jew's mallow leaves are close to the range in our wheat leaf samples; whereas moringa's reported PA is 300 % greater than the maximum value found in our wheat leaves. Ratios reported for these plants are much lower than our values, PA:Fe=0.48-2.79 and PA:Zn=3.48-10.98, with baobab's leaves being the exemption.

Similarly, our grain phytate ratios were affected significantly by P treatments. PA:Ca, PA:Zn and PAxCa:Zn, $p<0.001$ and PA:Fe $p<0.05$. Lower P supply treatments tended to give lower ratios. All the values calculated exceeded the critical values proposed for a good bioavailability of each mineral element. Even the lowest treatment, PA:Ca values exceeded the critical value 5.6-fold, PA:Fe was 20 times higher, PA:Zn values exceeded the critical 2.6-fold and PAxCa:Zn was 2.3 times higher.

As for the zinc experiment, PA:Zn ratios were influenced by the treatments, showing a decreasing tendency. PA:Zn ratios were high, treatments 1 and 2 had

ratios of 86 and 83 respectively, representing 5.7 times more than the recommended limit for good bioavailability. Then on treatments 3 and 4, we observed a 56.8 % decrease on T3 and a further 37 % on T4.

In summary, ratios were reduced from 86.01 to 22.58 (73 %). Wang (2015) also reported decreasing PA:Zn ratios with Zn application to soil, similar to ours, reductions of 14.1 %, 9.1 % and 27.3 % among the different treatments, authors reported they managed to reduce ratios above 30 to less than 10 with the highest Zn treatment. The decrease of PA:Zn molar ratios was caused mostly by the increase of Zn in the grain, contrary to what Erdal (2002) and Wang (2015) reported, our Zn treatments did not have any effect on the PA levels.

### 2.5.7 Phosphorus but not zinc treatments affected grain yield.

Results indicated that grain yield was affected by the P treatments; lowest treatment had the lowest concentration of PA, which increased about 75 % in treatment 3 and further decreased in T4 by 19.5 %. Treatment 3 achieved the highest grain yield as well. Grain yield was not affected by the zinc treatments p=0.637, similar to what Wang (2015) reported, and opposite to the studies of Cakmak (2010) and Kalayci (1999) stated in their studies.

We observed differences when the grain yield was considered to account for a dilution effect. Phytic acid concentrations in an ANOVA test generated two different clusters, the two lowest and the two highest, the main increase was observed from T2 to T3 by 96.4 %. These results are conflicting to the results given

by the “uncorrected” data, in which there were no differences between T2 and T3. Additionally, there were differences between the correlation coefficients, with the data recalculated, PA-Fe and PA-Co were no longer associated, PA-Cu, PA-Mg, PA-P and PA-Zn relationship strength decreased, PA-Ca strength increased and PA-K stayed the same.

### 2.5.8 Wrapping up: relationship of leaf and wholegrain PA and mineral concentrations

One of the aims of these experiments was to explore the possibility of using leaf mineral concentrations as a proxy for the content in the wholegrain. In the P experiment, our results indicated a strong relationship between the leaf and wholegrain concentrations of Mg (r=0.881) and P (r=0.829), a moderate relationship with Ca (r=0.558) and Zn (r=0.482) and a rather weak association for Mn (r=0.373) and PA (r=0.370).

PA in leaf and PA in wholegrain were only weakly associated (r=0.370) whereas leaf P and wholegrain PA had a moderate-high relationship (r=0.638).

Our results are similar to those reported by Raboy & Dickinson (1984) who on a previous experiment assessed the P status of the plants aiming to learn if it could be used to predict PA levels in mature seeds. Their results indicated a close relationship between P concentration and total P, PA and Pi with correlation coefficients ranging from 0.88-0.97. The authors significantly correlated phytic acid P in seed with total P in leaf (r=0.95). In our P experiment phytic acid-P in seed

had a strong positive relationship with total P in leaf (r=0.638 p<0.001, whereas no significant relationship was found for the Zn treatments where the phosphorus concentration was equivalent to treatment 3 (0.25 mmol $L^{-1}$).

In Raboy & Dickinson (1984) studies the seed Zn concentration closely reflected leaf Zn concentrations (r=0.96), in our study, we observed a significant but not that strong relationship in the P experiment (r=0.482 p<0.001) but it was slightly higher in the Zn experiment (r=0.658 p<0.001).

To explore deeper, the linear regressions for some of these relationships were run. The equations calculated for Ca, K, Mg, P, Zn, PA, PA-grain vs P-leaf were significant, p<0.001 and Mn p=0.003. Magnesium and phosphorus were the ones explaining more percentage of variability (77.5 and 68.1 %, respectively).

In our zinc experiment, there were some significant but rather weak associations between the concentrations in leaf and wholegrain such as Ca (r= -0.387), Co (r= -0.360), Cu (r=0.428) and K (r= -0.316). Zinc was the only one with a medium-high relationship (r=0.659). Moreover in this experiment, contrary to what was found with P treatments, P and PA had no relationship at all. This is perhaps explained by the fact that Zn did not affect P concentrations, as has been reported in previous findings where the effects of P treatment on plant and seed P and phytic acid were largely independent of the effects of Zn treatment on leaf and seed Zn (Raboy & Dickinson, 1984). In addition this could be because of the known antagonistic effect of Zn and P. Equally, linear regressions were run but most of

them described very little of the variance, so they were not further discussed. The equation for leaf-grain zinc described 42.5 % of the variance and was highly significant ($p<0.001$).

It is important to mention that the correlation analysis and linear regressions were run with pooled data, that is, treatments were not considered. In addition, multiple variable analysis were not performed. It would be interesting for future work, to analyse the effect of the treatments or multiple variable relationships, doing this could, perhaps provide a broader insight on the complexity of mineral relationships. Other interesting suggestions would be the analysis of other organs/tissues of the plant as well as different growing stages.

To our knowledge no literature is available reporting PA values in wheat leaves, probably because is of no commercial interest and/or has no use as feed. However, these findings might contribute to the understanding of phosphorus accumulation and storage in wheat as well as its interactions with other minerals.

In summary, our findings provide further support to nutrient culture as method to produce seed with varying levels of PA and provided data that can be used In future studies to model mineral interactions and PA concentrations.

# 3. ANALYSIS OF PHYTIC ACID CONCENTRATION IN LINES DERIVED FROM THE WATKINS DIVERSITY SET

## 3.1 INTRODUCTION

The search for new sources of genetic variability is a crucial step towards generating novel crops. There are numerous and unexploited traits, such as pest or drought resistance, which have been lost due to domestication in modern cultivars of wheat, but are inherent to landraces. Recently, a big set of landraces that was collected by A. E. Watkins (1928) from 34 countries, now called “Watkins collection” has been established as a valuable source for gene discovery. In this study, we analysed the phytic acid concentrations of 24 genotypes derived from this data collection.

Worldwide, about 800 million people are chronically hungry, meaning that they are undernourished in terms of calories (FAO et al., 2017). Malnutrition is linked across the life cycle, with undernutrition in foetal and early life contributing to both immediate and long-term health problems such as stunted physical growth, coronary heart disease, stroke, diabetes, and abdominal obesity, as well as economic costs due to loss of human capital (FAO et al., 2019).

More than 2 billion people are affected by hidden hunger, meaning that they suffer from micronutrient deficiencies (WHO & FAO, 2006). Hidden Hunger refers to a lack or loss of dietary quality that leaves individuals or populations with deficiencies in essential micronutrients which negatively impact on health, cognition, function, survival, and economic potential (Sight and Life Press, 2012).

Iron deficiency in childhood stunts cognitive development which hinders academic performance and future earnings potential as adults. Zinc deficiency adversely affects children and adults by weakening immune systems, increasing rates of childhood diarrhoea and pneumonia, and contributing to increased rates of childhood stunting. Globally, zinc deficiency contributes to 116,000 child deaths per year—a number that would be much higher if researchers were able to count the number of deaths caused by preterm births in zinc-depleted mothers (Food Fortification Initiative, 2019).

The major reason for micronutrient deficiency in the populations of the developing countries is the predominance of non-diversified cereal- and plant-based diets, which are poor in micronutrients, as compared to the meat rich diets of people in developed countries (Gómez-Galera et al., 2010; Grotz & Guerinot, 2006; Rawat et al., 2013). Anti-nutritional factors like phytic acid, fibres and tannins further reduce the bio-availability of these minerals from dietary intakes by preventing their absorption in the intestine (Cowieson et al., 2016; Hefferon, 2015; P. J. White & Broadley, 2009).

Engineering plants with enhanced mineral content could potentially alleviate current problems of human mineral deficiency by enhancing crop yields and/or fortifying plants pre-harvest (Grotz & Guerinot, 2006; Hefferon, 2015). Among dietary diversification, supplementation, fortification and biofortification of crop plants, the removal or reduction of phytic acid and other antinutrients has been considered as an approach for alleviating micronutrient malnutrition.

There have been enormous efforts to increase iron and zinc concentrations in grains but more often than not, these achievements will be hindered by the great amount of phytic acid in seeds.

Exploring the diversity of phytic acid and the phytic acid ratios will allow to identify varieties that could be used in breeding programs to improve the nutritional quality of wheat.

## 3.2 AIMS AND OBJECTIVES

To explore and describe the variability of phytic acid (PA) in wholegrain flour and white flour samples of wheat lines derived from Watkins landraces and assess the potential bioavailability of minerals such as Fe and Zn. This will allow to identify genotypes with low phytic acid that could be included in future breeding programs to reduce the antinutrient content in wheat and increase the mineral bioavailability. This study was performed with the following objectives: 1) determine PA concentrations in a wide set of genotypes derived from Watkins landraces; 2) quantify the effect of the genotype, environment and the G x E interaction on the phytic acid concentrations; 3) assess the potential bioavailability of minerals such as Fe and Zn by calculating phytic acid ratios.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Plant material

Previous to this study, a panel of 254 wheat genotypes derived from crosses between landraces, collected in the 1930's by A.E. Watkins (1928), and the UK spring wheat cultivar Paragon, were grown at Bunny Farm site of the University of Nottingham in 2015–16 and 2016–17 and at the Rothamsted Research farm in 2015–16 and 2017–18. Countries/regions of origin of these genotypes were: Australia, India, France, Morocco, Burma, Canary Islands, Spain, Cyprus, Turkey, Bulgaria, Portugal, Palestine, Greece, and Tunisia.

Marker analysis allowed a core set of 119 lines to be identified and 85 of these crossed with Paragon to develop F1 progeny, followed by four to six rounds of selfing (F4: F6) to develop populations of recombinant inbred lines (Winfield et al., 2018; Wingen et al., 2014). Each population comprises 94 lines, and 12 or 13 lines from 19 populations were selected to constitute the initial panel of 245 wheat genotypes. The lines were selected based on their performance (including yields) and adaptation to the UK (including flowering time) (Khokhar et al., 2020).

### 3.3.2 Experimental sites and management

The details of field experiments conducted at the University of Nottingham and Rothamsted Research, sites are described in Table 3-1.

Table 3-1 Field experiments details. Modified from Khokhar (2020).

| Site | Sowing | Harvesting | Harvest year | Grid | Previous crop |
|---|---|---|---|---|---|
| **(UoN)** | 05.04.2016 | 01.09.2016 | 1 | 52˚51’ 45” N | Oilseed rape |
| | 27.03.2017 | 24.08.2017 | 2 | 1˚7’ 30.6” W | Oilseed rape |
| **(RRes)** | 21.03.2016 | 1-2.09.2016 | 1 | 51˚48’ 18” N | Wheat |
| | 26.10.2017 | 21-22.08.2018 | 2 | 0˚ 23’ 01” W | Winter Oat |

UoN, University of Nottingham; RRes, Rothamsted Research.

The plots were arranged in a randomised factorial block design with three replications at both sites. The plot size was 1 $m^2$. To achieve a target population size of 200 plants $m^{-2}$, seed rates were selected on 1000 grain weight (TGW) basis and, ~350 seeds $m^{-2}$ were used per plot. Seeds were planted using a power drill after field preparation.

Nitrogen fertiliser at Nottingham was applied at the rate of 175 kg $ha^{-1}$ as Nitram (34.5 % N), one-third of total N was applied at sowing time and the remaining was applied at growth stage 31, onset of stem extension (GS31) in 2015–16 and 2016–17. At Rothamsted nitrogen fertilisers were applied in two doses; first dose at the rate of 222 kg $ha^{-1}$ as double top (27 % N) and 2nd dose at a rate of 101 kg $ha^{-1}$ as ammonium nitrate (34.5% N) in 2015–16 while in 2017–18 only a single dose of ammonium nitrate was applied at the rate of 370 kg $ha^{-1}$. All the plots were

managed and protected from pest, diseases, and weeds by using herbicides and pesticides to ensure to get the maximum yield. Crops were harvested at maturity by a small combine harvester, Khokhar (2020).

### 3.3.3 Grain sampling and digestion of 245 wheat genotypes

Ears were harvested from an area of 0.5-meter length from central rows of each plot, dried at 80˚C for 48 hours in the oven and then threshed mechanically. Approximately 10 grains of all 245 wheat genotypes in three replicates from Nottingham (n = 735) and in one replicate from Rothamsted site (n = 245) in 2015–16 were weighed and the grain samples were soaked in 3 mL 70% Trace Analysis Grade (TAG) $HNO_3$ and 2 mL hydrogen peroxide ($H_2O_2$), overnight at room temperature and were used for the microwave digestion. Refer to Khokhar (2020) for full technical details of the microwave digestion.

### 3.3.4 Brief description of the initial panel of 245 genotypes

The concentration of 31 mineral elements was determined and analysed by ICP-MS by Khokhar (2020). For the purpose of this study we focused on the minerals that are most associated with phytic acid: Ca, Fe, K, P and. Table 3-2 presents descriptive statistics of the initial panel.

Table 3-2 Mineral element concentrations of Watkins initial panel of 245 genotypes. Data are in mg $kg^{-1}$. From Khokhar (2020), data for Ca, K and P are unpublished data.

| Element | n | Mean | SD | Median | Range | LOD |
|---|---|---|---|---|---|---|
| **Ca** | 716 | 429.9 | 96.1 | 429.2 | 17.9 - 749.9 | 35.701 |
| **Fe** | 715 | 35.0 | 7.0 | 34.3 | 2.1 - 71.2 | 4.184 |
| **K** | 716 | 4949.9 | 742.5 | 4945.8 | 53 – 7729.6 | 7.122 |
| **P** | 716 | 4707.1 | 724.2 | 4745.3 | 47.6 – 7284.9 | 3.519 |
| **Zn** | 716 | 31.5 | 6.9 | 30.6 | 0.7- 60.1 | 1.315 |

SD, Standard Deviation; n, number of values; LOD, Limit of Detection.

From this 245 genotype panel, 24 genotypes were selected and divided into four levels according to their Fe and Zn concentrations, High_Fe - High_Zn, Low_Fe - High_Zn, High_Fe-Low_Zn and Low_Fe-Low_Zn. This panel of 24 genotypes was used in the phytic acid determinations, Figure 3-1.

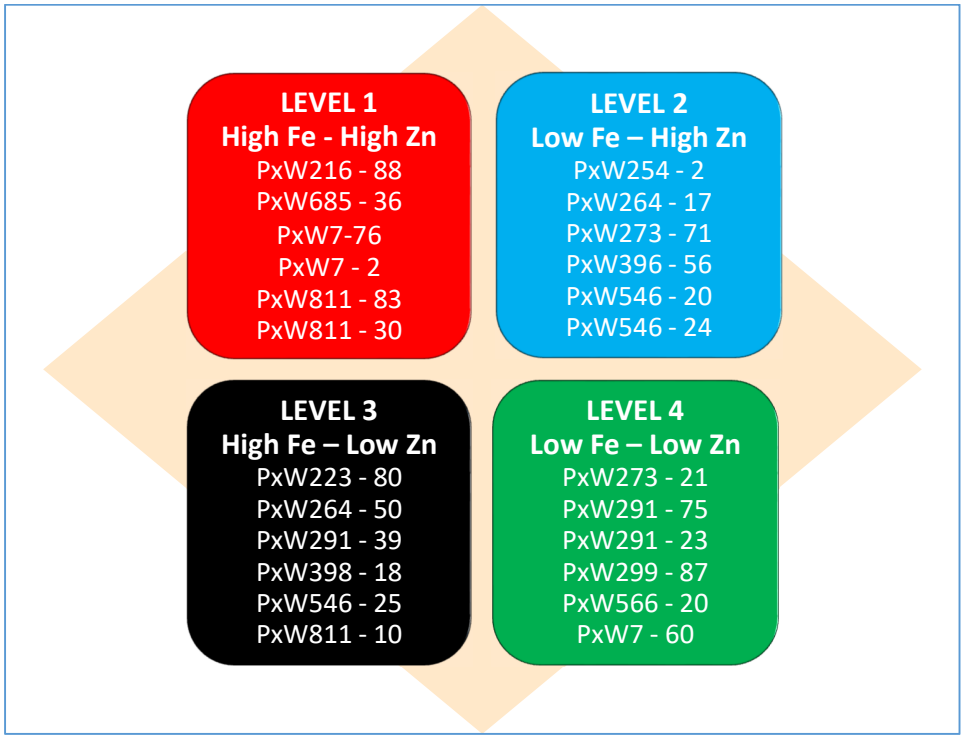



Figure 3-1 Panel of 24 genotypes used for phytic acid determination.

Table 3-3 Subset of 24 genotypes according to their country of origin and level of Fe-Zn concentration in a panel of 245 genotypes of wholegrain flour wheat.

| | HFe-HZn | LFe-HZn | HFe-LZn | LFe-LZn |
|---|---|---|---|---|
| **Australia** | PxW7 – 2<br>PxW7 - 76 | | | PxW7 - 60 |
| **Burma** | | | PxW223 - 80 | |
| **Canary Islands** | | PxW264 - 17 | PxW264 - 50 | |
| **Cyprus** | | | PxW291 - 39 | PxW291 – 23<br>PxW291 - 75 |
| **Greece** | | | | PxW566 - 20 |
| **Morocco** | PxW216 - 88 | PxW254 - 2 | | |
| **Palestine** | | | PxW398 - 18 | |
| **Portugal** | | PxW396 - 56 | | |
| **Spain** | PxW685 - 36 | PxW273 – 71<br>PxW546 – 20<br>PxW546 - 24 | PxW546 - 25 | PxW273 - 21 |
| **Tunisia** | PxW811 – 30<br>PxW811 - 83 | | PxW811 - 10 | |
| **Turkey** | | | | PxW299 - 87 |

HFe-HZn=Level 1, High Fe-High Zn (red); LFe-HZn=Level 2, Low Fe-High Zn (blue); HFe-LZn=Level 3, High Fe-Low Zn (black); LFe-LZn=Level 4, Low Fe-Low Zn (green). Data from Khokhar (2020).

### 3.3.5 Milling and digestion of a sub-set of 24 wheat genotypes

Based on the concentrations of grain Zn and Fe in the wholegrain flour samples of a panel of 245 wheat genotypes grown at Nottingham and Rothamsted in 2015–16, a sub-set of 24 genotypes was selected to capture the diversity present in the panel, Table 3-3. Grain samples of these genotypes were resampled from three replicate plots per line from Nottingham in 2015–16, and from freshly harvested plots in 2016–17, and resampled from three replicate plots per line from

Rothamsted in 2015–16, and from freshly harvested plots in 2017–18. Samples were dried at 40˚C overnight before milling, 6 g subsample of grain were then milled at 30 rpm for 30 seconds using a Laboratory Flour Mill AQC 806 (Agromatic AG, Laupen, Switzerland) to obtain wheat white flour. Wholegrain flour samples (n = 129) of these genotypes, resampled from the Nottingham and Rothamsted 2015–16 material, were digested in a similar manner as the 245 wheat genotypes with a soaking step. The subsample (~0.300 g DW) of wheat white flour samples (n = 129) were digested without soaking step. The wholegrain flour (n = 114) and white flour (n = 114) samples of these sub-set of genotypes from Nottingham in 2016–17 and from Rothamsted in 2017–18 were digested using a microwave system. The wholegrain flour samples were soaked in in 6 mL 70 % TAG $HNO_3$ solution for one hour at room temperature. Refer to Khokhar (2020) for full technical details of the microwave digestion.

### 3.3.6 Wholegrain (WGF) and white flour (WF) mineral analysis

Wholegrain and white flour concentration mineral concentrations were determined by ICP-MS (Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany). Internal and external multielemental calibration standards were included for quantification purposes. In total 980 wholegrain flour samples of a panel of 245 wheat genotypes, 735 from Nottingham and 245 from Rothamsted harvested in 2015–16 were analysed. In addition, 129 wholegrain flour and 129 wheat white flour samples of a sub-set of 24 genotypes were

analysed from both sites in 2015–16. In 2016–17, 114 wholegrain flour and 114 wheat white flour samples of the sub-set of 24 genotypes were analysed from both sites. Grain samples of five genotypes in the sub-set of 24 genotypes were not available from Rothamsted in the first-year harvest and from both sites in the second-year harvest. The respective Zn and Fe recovery from CRMs were 97.5 % and 81 % for samples from 2015–16, and 96.7 % and 112.2 % for samples from 2016–17. The respective LOD for Zn and Fe were 0.57 and 3.14 mg $kg^{-1}$ for samples from 2015–16, and 1.71 and 4.88 mg $kg^{-1}$ for samples from 2016–17. Refer to Khokhar (2020) for full technical details of the ICP-MS analysis.

### 3.3.7 Descriptive statistics of mineral elements of the 24 genotypes panel

The following tables present descriptive statistics for Phosphorus, Zinc and Iron of the 24 genotypes used for phytic acid determination. Harvest year 1 in Table 3-4 and harvest year 2 in Table 3-5.

Table 3-4 Phosphorus, zinc and iron concentration in white flour and wholegrain flour of the panel of 24 genotypes in harvest year 1. From Khokhar (2020); P data unpublished.

| | Element | Site | n | Mean | Median | Min | Max | SD |
|---|---|---|---|---|---|---|---|---|
| **White flour** | P | RRes | 57 | 1328.2 | 1312.3 | 994.6 | 1645.1 | 154.51 |
| | | UoN | 72 | 1421.3 | 1409.1 | 984.3 | 1984.9 | 206.41 |
| | Zn | RRes | 57 | 12.4 | 12.6 | 6.9 | 19.3 | 2.50 |
| | | UoN | 72 | 10.9 | 10.4 | 6.4 | 18.4 | 2.65 |
| | Fe | RRes | 57 | 15.6 | 14.5 | 6.6 | 50.8 | 6.98 |
| | | UoN | 72 | 17.1 | 15.6 | 6.7 | 34.1 | 6.47 |
| **Wholegrain flour** | P | RRes | 57 | 4139.8 | 4066.1 | 3241.3 | 5305.5 | 432.58 |
| | | UoN | 72 | 4471.0 | 4460.0 | 3492.5 | 5732.2 | 427.50 |
| | Zn | RRes | 57 | 38.8 | 39.4 | 26.5 | 64.8 | 7.64 |
| | | UoN | 72 | 30.0 | 28.7 | 19.1 | 55.7 | 7.32 |
| | Fe | RRes | 57 | 29.9 | 27.8 | 21.1 | 45.7 | 5.99 |
| | | UoN | 72 | 31.7 | 30.9 | 21.3 | 47.2 | 6.04 |

Values obtained by ICP-MS multielemental analysis. RRes, Rothamsted Research; UoN, University of Nottingham; n, number of values; Min, minimum value; Max, maximum value; SD, standard deviation.

Table 3-5 Phosphorus, zinc and iron concentration in white flour and wholegrain flour of the panel of 24 genotypes in harvest year 2. From Khokhar et al., (2020); P data is unpublished.

| | Element | Site | n | Mean | Median | Min | Max | SD |
|---|---|---|---|---|---|---|---|---|
| **White flour** | P | RRes | 57 | 1093.9 | 1077.8 | 929.8 | 1411.1 | 106.81 |
| | | UoN | 57 | 1171.7 | 1170.8 | 907.3 | 1520.6 | 137.37 |
| | Zn | RRes | 57 | 13.0 | 12.6 | 7.4 | 19.6 | 2.61 |
| | | UoN | 57 | 7.7 | 7.5 | 4.4 | 14.7 | 2.08 |
| | Fe | RRes | 57 | 10.6 | 10.3 | 7.2 | 16.4 | 1.74 |
| | | UoN | 57 | 11.3 | 11.0 | 5.4 | 18.0 | 2.72 |
| **Wholegrain flour** | P | RRes | 57 | 3658.9 | 3737.4 | 2804.4 | 4352.6 | 373.92 |
| | | UoN | 57 | 3953.1 | 3875.5 | 3031.7 | 4683.9 | 391.91 |
| | Zn | RRes | 57 | 40.5 | 41.0 | 25.7 | 56.6 | 7.62 |
| | | UoN | 57 | 23.1 | 23.2 | 15.2 | 38.4 | 4.53 |
| | Fe | RRes | 57 | 44.8 | 45.6 | 32.0 | 54.9 | 5.91 |
| | | UoN | 57 | 36.2 | 35.3 | 22.5 | 51.4 | 5.61 |

Values obtained by ICP-MS multielemental analysis. RRes, Rothamsted Research; UoN, University of Nottingham; n, number of values; Min, minimum value; Max, maximum value; SD, standard deviation.

### 3.3.8 Phytic acid determination in wholegrain (WGF) and white flour (WF) of the 24 genotypes sub-set

Phytic acid determinations were made using the above described sub-set of 24 genotypes. To obtain the wholegrain flour (WGF), 5 g subsample was milled at 30 rpm for 30 seconds using a Laboratory Flour Mill (AQC7806, Agromatic AG; Laupen, Zurich, Switzerland), Figure 3-2, without the drum sieve (250 µm). Care was taken to clean all parts of the mill after each sample using a vacuum machine. Whenever the grain material was scarce, only 3 g were taken to obtain the wholegrain flour. To obtain the white flour, samples were processed as described in section 3.3.5 in page 170, and from the resulting powder 1 g was taken to be used in the phytic acid determinations.

Figure 3-2 Laboratory mill AQC806.

The wholegrain and white flour phytic acid contents were determined using a commercial kit (Phytic acid/total phosphorus; Megazyme International Ireland, Bray Co. Wicklow, Ireland), with slight modifications. The assay measures phytic acid as phosphorus released by a phytase and an alkaline phosphatase. The

procedure was done as described in section 2.3.12 in page 78. The principle of the method is presented in Figure 2-10 in page 79.

### 3.3.9 Phytic acid data cleaning

For phytic acid determination, a total of 129 samples of wholegrain flour and 129 samples of white flour were processed in 14 runs for harvest year 1, see Table 3-1 for detailed dates and similarly, for harvest year 2, 113 samples of wholegrain flour and 113 samples of white flour were analysed in 14 runs, see Table 3-1 for detailed dates.

Raw data was cleaned and processed as described in the subsequent paragraphs.

Firstly, the difference in absorbance (delta-Abs) values of each blank, 12 and 15 data points for harvest year 1 and 2 respectively, were averaged and the LOD was reported as three times the standard deviation (SD). Two data points were excluded in harvest year 1. Table 3-6 below and Table 9-15 in Appendices.

Table 3-6 Summary of blanks data for limit of detection.

| | n | Mean delta-Abs | SD | LOD (SD * 3) | Phosphorus (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|
| **Harvest year 1** | 12 | 0.0219 | 0.0047 | 0.0140 | 89.0 | 315.0 |
| **Harvest year 2** | 15 | 0.0232 | 0.0052 | 0.0156 | 100.75 | 357.27 |

Table shows the mean of the delta-Abs readings from blanks used to calculate the LOD. Delta-Abs is the difference in absorbance and is obtained by subtracting the absorbance values of the free phosphorus samples from the absorbance values of total phosphorus samples as indicated by the instructions of the manufacturer. LOD, limit of detection; n, number of values; SD, standard deviation; delta-Abs, delta absorbance.

Data points lower than the LODs were substituted by 50 % the LOD. In harvest year 1, only one data point had to be replaced. In harvest year 2, 54 data points were substituted, all corresponded to white flour samples.

To identify any possible outliers, we calculated the LOQ (limit of quantification), defined as the global mean plus five times the standard deviation. Samples above this value were removed from further analysis. The LOQ calculated in harvest year 1 were 4198.81 and 18569.29 mg $kg^{-1}$ for white flour and wholegrain flour samples respectively and in harvest year 2, 1860.36 and 15847 mg $kg^{-1}$ for white flour and wholegrain flour respectively. No data points were excluded in either year. For details, see Table 3-7 below and Table 9-16 in Appendices.

Table 3-7 Phytic acid in white flour and wholegrain flour. Data are in mg $kg^{-1}$.

| Sample type | Harvest year | Mean | SD | LOQ |
|---|---|---|---|---|
| White flour | 1 | 1430.1 | 553.8 | 4198.8 |
| White flour | 2 | 414.7 | 289.1 | 1860.4 |
| Wholegrain flour | 1 | 9594.5 | 1795.0 | 18569.3 |
| Wholegrain flour | 2 | 9122.3 | 1344.9 | 15847.0 |

SD, Standard deviation; LOQ, limit of quantification.

### 3.3.10 Phytic acid to mineral molar ratio determination

Data from mineral concentrations previously generated, Khokhar (2020), was used to calculate phytic acid ratios. Millimolar ratios were obtained first calculating the millimoles of PA, by dividing the concentration in mg $kg^{-1}$ by its

molecular weight (MW) in g $mol^{-1}$ and equally with each of the mineral elements. Next, the resulting millimoles of PA were divided by the millimoles of the corresponding mineral. Molecular weights used were PA=660.04, Ca=40.08, Fe=55.85, P=30.97 and Zn=65.4 g $mol^{-1}$.

### 3.3.11 Statistical analysis

Data management and analysis were performed using GenStat 19th Edition (VSN International Ltd, Hemel Hempstead, UK), Microsoft Excel 2016 and Sigma Plot Version 13.0 (Systat Software, Inc.).

## 3.4 RESULTS

The recovery of the assay was calculated according to the values obtained by the oat flour reference material provided by the manufacturer. It was extracted from 1 g of oat flour in 20 mL of extraction solution and processed in each run as the rest of the samples. The established concentration stated by the manufacturer is 4990 mg $kg^{-1}$ of phosphorus which is equivalent to 17710 mg $kg^{-1}$ of phytic acid.

The mean value for the oat reference material in harvest year 1 (n=14) was 2991.21 mg $kg^{-1}$ of phosphorus, equivalent to 10607.14 mg $kg^{-1}$ of phytic acid which accounted for a recovery of 59.89 % and a relative standard deviation (RSD %) of 20.51 %. In harvest year 2 the average was 2129.76 mg $kg^{-1}$ of phosphorus, equivalent to 7552.34 mg $kg^{-1}$ of phytic acid which accounted for a recovery of 42.64 % and RSD= 23.06 %. RSD was calculated dividing the standard deviation by the mean value. See Table 9-17 for all raw data.

### 3.4.1 Results phytic acid concentrations

The phytic acid mean of a panel of 24 genotypes in white flour samples was 1430.1 ± 553.8 mg $kg^{-1}$ (n=129) in both sites in harvest year 1 and 414.7 ± 289.1 mg $kg^{-1}$ in harvest year 2 (n=113) also in both sites. For the wholegrain flour fraction the mean values were 9594 ± 1795 mg $kg^{-1}$ in harvest year 1 (n=129) and 9122 ± 1345 mg $kg^{-1}$ (n=113) in harvest year 2 in both sites (mean ± SD).

Mean phytic acid concentration for both harvest years in white flour were higher at UoN site, 1604.3 ± 597.7 mg $kg^{-1}$ (n=72), 1210.0 ± 399.6 mg $kg^{-1}$ (n=57),

compared to RRes site. In harvest year 2, mean concentrations were 504.1 ± 342.5 mg $kg^{-1}$ (n=56) at UoN site and 327.0 ± 190.1 (n=57) at RRes; means ± SD.

For wholegrain flour samples, mean PA was higher at UoN site in harvest year 1, 10294 ± 1571 mg $kg^{-1}$ compared to RRes site, 8710 ± 1676 mg $kg^{-1}$. Whereas in harvest year 2, the mean PA was higher at RRes site, 9397 ± 1353 mg $kg^{-1}$ compared to the UoN site, 8843 ± 1289 mg $kg^{-1}$, means ± SD; Figure 3-3.

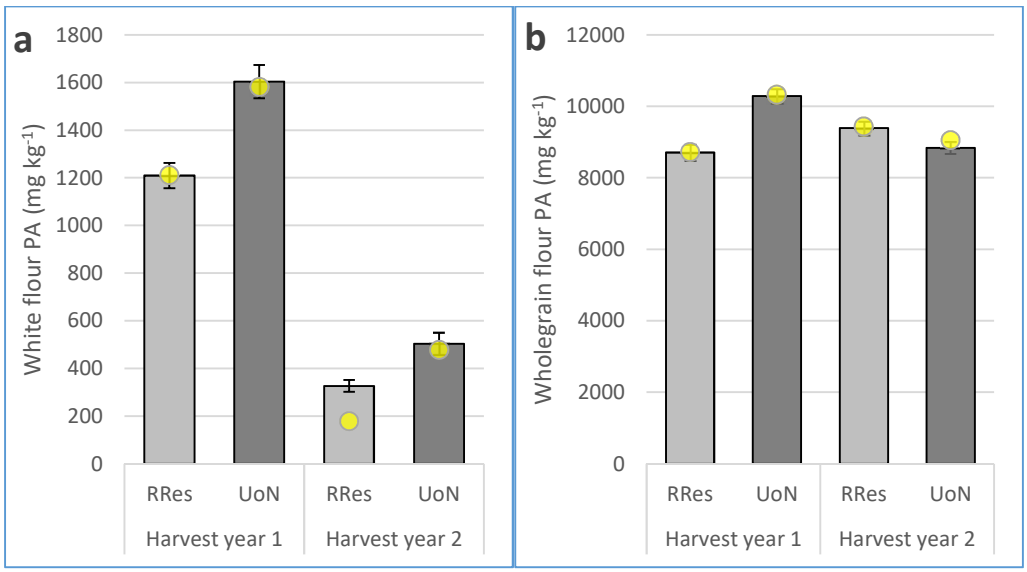



Figure 3-3 White flour (a) and wholegrain flour (b) phytic acid (PA) concentration of a panel of 24 genotypes derived from Watkins landraces grown in two sites in the UK. Mean ± standard error bars (SE). Yellow dots represent the median. RREs= Rothamsted Research, UoN= University of Nottingham.

In white flour samples, the genotype with the lowest concentration of phytic acid in harvest year 1 was PxW299 – 87 with a mean of 876.4 mg $kg^{-1}$ (mean of both sites) and in harvest year 2, the lowest concentration corresponded to the genotype PxW291 – 75 with 213.6 mg $kg^{-1}$ (mean of both sites). The PA

concentration of three genotypes (PxW811 – 83, PxW264 – 50 and PxW811 – 30) was under the LOD value. The highest concentration was obtained from the genotype PxW546 – 25 with a mean of 2682.6 mg kg (mean of both sites) in harvest year 1 and PxW546 – 20 in harvest year 2 with 845.9 mg kg (mean of both sites).

For wholegrain flour samples, the lowest mean value of phytic acid was in the genotype PxW566 – 20 with 8112 mg $kg^{-1}$ in harvest year 1 and PxW7 – 60 in harvest year 2 with a mean of 7145 mg $kg^{-1}$. The highest value in harvest year 1 was in PxW546 – 24 with 10918 mg $kg^{-1}$ whereas in harvest year 1 was PxW685 – 36 with 10551 mg $kg^{-1}$. The mean, minimum and maximum concentration values by site and year can be found in Table 3-8 and by genotype in Table 3-9.

Table 3-8 Phytic acid concentration values in white flour (WF) and wholegrain flour (WGF) by site and harvest year. Data are in mg $kg^{-1}$.

| Site | n | | Mean | | Min | | Max | | SD | |
|---|---|---|---|---|---|---|---|---|---|---|
| | WF | WGF | WF | WGF | WF | WGF | WF | WGF | WF | WGF |
| **Harvest year 1** | | | | | | | | | | |
| **RRes** | 57 | 57 | 1210 | 8710 | 545.1 | 4853 | 2135 | 11995 | 399.6 | 1676 |
| **UoN** | 72 | 72 | 1604.3 | 10294 | 774.0 | 6425 | 3071 | 13220 | 597.7 | 1571 |
| **Harvest year 2** | | | | | | | | | | |
| **RRes** | 57 | 57 | 327 | 9397 | 347.7 | 6713 | 1024 | 12333 | 190.1 | 1353 |
| **UoN** | 56 | 56 | 504.1 | 8843 | 391.2 | 5558 | 1564 | 11434 | 342.5 | 1289 |

RRes, Rothamsted Research; UoN, University of Nottingham; SD, Standard deviation; Min, minimum value; Max, maximum value.

Table 3-9 Mean phytic acid concentrations by genotype and site.

| | | UoN – HY1 | | UoN - HY2 | | RRes - HY1 | | RRes - HY2 | |
|---|---|---|---|---|---|---|---|---|---|
| **Genotype** | **Level** | **WF** | **WGF** | **WF** | **WGF** | **WF** | **WGF** | **WF** | **WGF** |
| PxW216 - 88 | H_Fe-H_Zn | 2478.5 | 11633.0 | 701.9 | 9116.7 | 1558.6 | 9558.3 | 459.5 | 10664.1 |
| PxW223 - 80 | H_Fe-L_Zn | 1839.9 | 10022.8 | | | | | | |
| PxW254 - 2 | L_Fe-H_Zn | 1639.4 | 11698.9 | 699.2 | 9690.8 | 1539.7 | 9725.2 | 178.6 | 8170.3 |
| PxW264 - 17 | L_Fe-H_Zn | 1439.8 | 8941.8 | 367.7 | 9095.3 | 1028.4 | 7598.4 | 468.4 | 10187.6 |
| PxW264 - 50 | H_Fe-L_Zn | 827.4 | 10887.4 | 178.6 | 9254.8 | 1121.7 | 9316.0 | 178.6 | 8999.4 |
| PxW273 - 21 | L_Fe-L_Zn | 1757.6 | 8994.1 | 412.1 | 8515.9 | 1512.6 | 9339.8 | 458.0 | 9660.9 |
| PxW273 - 71 | L_Fe-H_Zn | 1435.3 | 11486.5 | 475.5 | 9244.4 | 1288.3 | 6882.1 | 178.6 | 8429.0 |
| PxW291 - 23 | L_Fe-L_Zn | 1257.4 | 8807.6 | 397.1 | 8953.1 | 952.1 | 7957.1 | 392.4 | 9219.3 |
| PxW291 - 39 | H_Fe-L_Zn | 1171.6 | 10382.9 | | | | | | |
| PxW291 - 75 | L_Fe-L_Zn | 1453.6 | 9921.3 | 178.6 | 6722.5 | 636.0 | 8088.9 | 248.5 | 10481.4 |
| PxW299 - 87 | L_Fe-L_Zn | 892.1 | 10242.7 | 322.1 | 8962.8 | 860.6 | 8938.9 | 178.6 | 7131.9 |
| PxW396 - 56 | L_Fe-H_Zn | 2362.2 | 8879.6 | 933.2 | 7285.5 | 1742.1 | 8611.3 | 684.8 | 10097.9 |
| PxW398 - 18 | H_Fe-L_Zn | 1476.8 | 10453.1 | | | | | | |
| PxW546 - 20 | L_Fe-H_Zn | 2432.9 | 11400.8 | 1284.2 | 8803.3 | 1709.5 | 7109.2 | 407.6 | 9091.9 |
| PxW546 - 24 | L_Fe-H_Zn | 1752.1 | 12080.1 | 811.4 | 8185.9 | 1110.1 | 9755.8 | 494.0 | 10155.8 |
| PxW546 - 25 | H_Fe-L_Zn | 2682.6 | 9743.9 | | | | | | |
| PxW566 - 20 | L_Fe-L_Zn | 1944.4 | 9087.2 | 669.3 | 8076.8 | 1055.7 | 7136.9 | 460.9 | 8617.6 |
| PxW685 - 36 | H_Fe-H_Zn | 1210.8 | 11045.1 | 374.1 | 10789.8 | 1096.6 | 8633.8 | 178.6 | 10311.5 |
| PxW7 - 2 | H_Fe-H_Zn | 1891.0 | 11477.6 | 679.5 | 10598.4 | 1687.2 | 9871.5 | 368.5 | 10359.2 |
| PxW7 - 60 | L_Fe-L_Zn | 1714.8 | 9338.3 | 378.3 | 6123.3 | 1208.8 | 7388.6 | 251.9 | 8167.4 |
| PxW7 - 76 | H_Fe-H_Zn | 1901.0 | 9900.4 | 459.8 | 9708.1 | 1013.3 | 10765.0 | 267.1 | 10972.5 |
| PxW811 - 10 | H_Fe-L_Zn | 930.7 | 9410.9 | | | | | | |
| PxW811 - 30 | H_Fe-H_Zn | 1023.6 | 10332.9 | 178.6 | 9361.2 | 965.8 | 9515.7 | 178.6 | 9451.0 |
| PxW811 - 83 | H_Fe-H_Zn | 986.7 | 10897.4 | 178.6 | 9303.8 | 903.2 | 9304.5 | 178.6 | 8374.6 |
| n | | 72 | 72 | 56 | 56 | 57 | 57 | 57 | 57 |
| Mean (mg $kg^{-1}$) | | 1604.3 | 10294 | 504.1 | 8843 | 1210 | 8710 | 327 | 9397 |

UoN, University of Nottingham; RRes, Rothamsted Research; WF, White flour; WGF, Wholegrain flour; HY1, Harvest year 1; HY2, Harvest year 2; n, number of values. Colours are levels as described previously in Figure 3-1: red, High_Fe - High_Zn; blue, Low_Fe - High_Zn; black, High_Fe-Low_Zn and green, Low_Fe-Low_Zn. Data are in mg $kg^{-1}$.

There was more variation (CV %) in white flour for PA concentration than in wholegrain flour at both RRes and UoN sites. Comparing between sites, RRes had a greater variation.

Results of the analysis of variance revealed significant genotype ($p<0.001$, LSD 1 %= 368.3), site ($p<0.001$, LSD 1 %=95.29) and year ($p<0.001$, LSD 1 %=95.33). In wholegrain samples the analysis showed significant genotype ($p<0.001$, LSD 1 %=1688), site ($p=0.001$, LSD 1 %=436.7) and year ($p=0.017$, LSD 1 %=331.1).

The environment was the greatest driver of PA variance in WF (55 %), more than twice than what was found in wholegrain (15 %). The G x E interaction was the greatest contributor in explaining variation in WGF (23 %) compared to WF (7 %). The genotype accounted for a greater proportion in WF compared to WGF and the residual term was greater for WGF (40 %) compared to WF (11 %), which could be due to variations in measurements or it could indicate that other variables not measured in this study are affecting the PA concentrations in WGF, Table 3-10.

Table 3-10 Effects of genotype, environment and their interaction (G x E) expressed as % of the total sum of squares from ANOVA analysis for phytic acid in white flour and wholegrain flour of a panel of 24 genotypes grown in the UK at two sites during two harvest years. Environment is the combination of site-year.

| | **PA white flour** | | | | |
|---|---|---|---|---|---|
| **Source of variation** | **df** | **SS** | **SS (%)** | **MS** | **p** |
| **Genotype** | 23 | 30186355 | 27.3 | 1312450 | <0.001 |
| **Environment** | 3 | 60765493 | 54.9 | 20255164 | <0.001 |
| **G x E** | 54 | 7714923 | 7.0 | 142869 | 0.001 |
| **Residual** | 161 | 12041480 | 10.9 | 74792 | |
| **Total** | 241 | 110708250 | | 459370 | |
| SE = 273.5 | CV = 28.61 % | Adj. $R^2$ = 84 % | | | |
| | **PA wholegrain flour** | | | | |
| **Source of variation** | **df** | **SS** | **SS (%)** | **MS** | **p** |
| **Genotype** | 23 | 135986532 | 21.6 | 5912458 | <0.001 |
| **Environment** | 3 | 96400557 | 15.3 | 32133519 | <0.001 |
| **G x E** | 54 | 142135233 | 22.6 | 2632134 | 0.008 |
| **Residual** | 161 | 253900733 | 40.4 | 1577023 | |
| **Total** | 241 | 628423055 | | 2607565 | |
| SE = 1256 | CV = 13.4 % | Adj. $R^2$ = 40 % | | | |

df, degrees of freedom; SS, sum of squares; SS (%), SS as percentage to the total; MS, mean squares; p, probability; SE, standard error; Adj. $R^2$, adjusted $R^2$ (%); CV: coefficient of variation (%).

### 3.4.2 Phytic acid-P as a percentage of total phosphorus

Phytic acid-P as a percentage of total P (phytic acid-P/Total P %) was calculated for white flour and wholegrain flour samples. The calculation was done dividing the phytic acid-P value (in the Megazyme kit it is referred as “Phosphorus”) by the total phosphorus (the value obtained by ICP-MS) and expressing it as a percentage.

The overall mean for both years and both sites in white flour (n=235) was 19.61 ± 11.83 % and varied between 4.07 and 51.55 %, the median was 18.36 %.

Wholegrain flour samples (n=240) had a mean of 65 ± 10.14 % and varied between 35.22 – 102.6 %, the median was 65.32 (mean ± SD).

The results indicated that in year 1 harvest the mean values were higher when compared to the results in year 2 harvest, 25.03 (range 11.7 – 41.15 %) and 31.43 % (range 4.49 – 51.55 %) in RRes and UoN respectively, compared to 8.17 (range 4.07 – 20.47 %) and 11.54 % (range 4.08 – 29 %) in RRes and UoN respectively. Overall, UoN site had significantly higher values ($p<0.001$) compared to RRes site (22.5 and 16.3 %, UoN and RRes respectively) in WF, while there were no differences between the sites in WGF.

Similar to PA, there was more variation (CV %) in white flour for phytic acid-P/Total P %, than in wholegrain flour at both RRes and UoN sites. Comparing between sites, RRes had a greater variation.

Phytic acid-P/total P % values in wholegrain flour were for RRes in harvest year 1, 59.48 % (range 35.22 – 89.58 %) and in harvest year 2, 72.51 % (range 55.37 – 102.65). At UoN site in harvest year 1, 64.96 % (range 45.12 – 87.35 %) and in harvest year 2, 63.08 % (range 47 – 86.63 %), Figure 3-4.

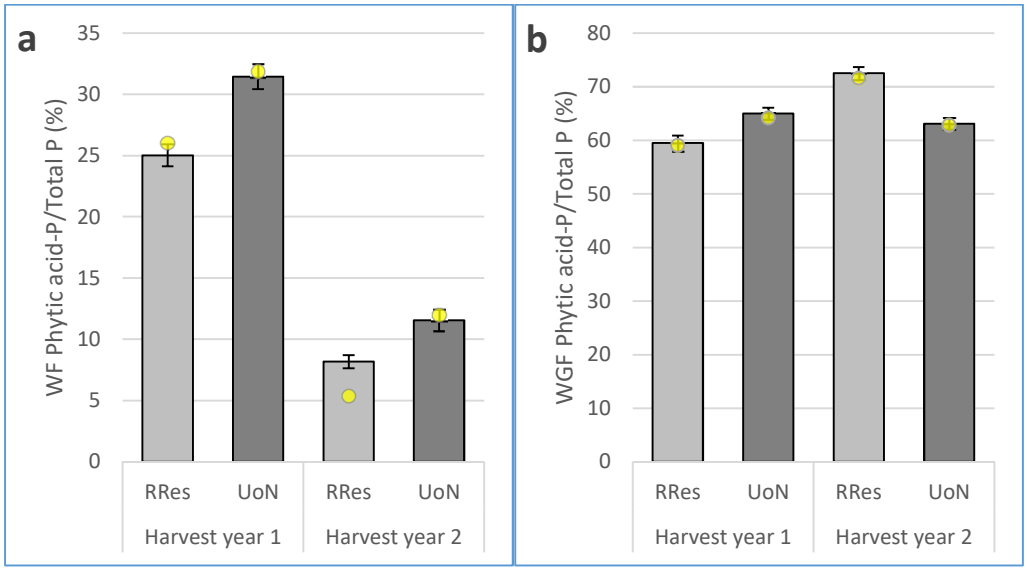



Figure 3-4 Phytic acid-P/total P % in, a. white flour (WF) and b. wholegrain flour (WGF) samples of a panel of 24 genotypes grown in two sites in the UK. Data are mean values ± standard error bars (SE). The yellow dot represents the median. RRes, Rothamsted Research; UoN, University of Nottingham.

Unbalanced ANOVA analysis revealed significant genotype ($p<0.001$, LSD 1 %=6.948), site ($p<0.001$, LSD 1 %=1.812) and year ($p<0.001$, LSD 1 %=1.811) for white flour phytic acid-P/total P %.

For wholegrain flour the unbalanced ANOVA revealed significant genotype ($p<0.030$, LSD 5 %=9.37) and year ($p<0.001$, LSD 1 %=3.204) for phytic acid-P/total P %. Figure 3-5 shows a comparison of the phytic acid-P/total P % in white and wholegrain flour by genotype.

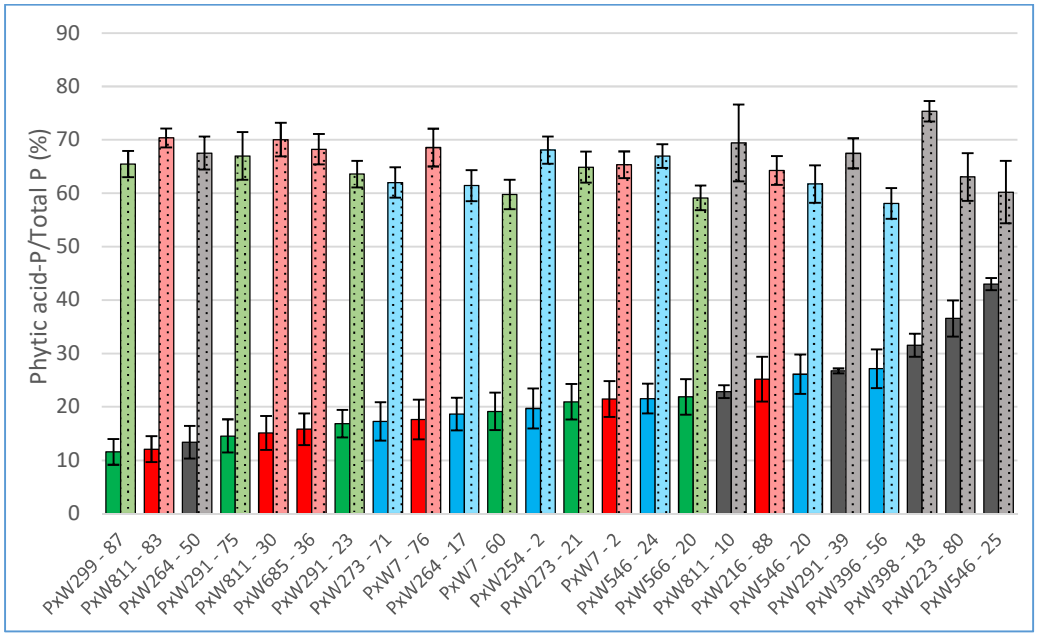



Figure 3-5 Phytic acid-P/total P % of a panel of 24 genotypes. White flour (solid bar) and wholegrain flour (patterned bar). Bars are means ± standard error bars (SE). Bars are ranked according to white flour concentrations. Colours correspond to levels of Fe-Zn as described in Figure 3-1.

The major source of variance in phytic acid-P/total P % in both WF and WGF was due to the environment but with big differences (60 and 21 %, WF and WGF respectively). The genotype had more effect than the G x E interaction in WF, while the opposite occurred in WGF, Table 3-11.

Table 3-11 Effects of genotype, environment and their interaction (G x E) expressed as % of the total sum of squares from ANOVA analysis for phytic acid-P/total % in white flour and wholegrain flour of a panel of 24 genotypes grown in the UK at two sites during two harvest years. Environment is the combination of site-year.

| | **Phytic acid-P/total %, white flour** | | | | |
|---|---|---|---|---|---|
| **Source of variation** | **df** | **SS** | **SS (%)** | **MS** | **p** |
| **Genotype** | 23 | 7523.45 | 23.0 | 327.11 | <0.001 |
| **Environment** | 3 | 19596.2 | 59.8 | 6532.07 | <0.001 |
| **G x E** | 54 | 1812.74 | 5.5 | 33.57 | 0.082 |
| **Residual** | 154 | 3839.81 | 11.7 | 24.93 | |
| **Total** | 234 | 32772.2 | | 140.05 | |
| SE = 4.993 | CV = 25.47 % | Adj. $R^2$ = 82 % | | | |
| | **Phytic acid-P/total %, wholegrain flour** | | | | |
| **Source of variation** | **df** | **SS** | **SS (%)** | **MS** | **p** |
| **Genotype** | 23 | 3413.88 | 13.9 | 148.43 | 0.006 |
| **Environment** | 3 | 5175.16 | 21.1 | 1725.05 | <0.001 |
| **G x E** | 54 | 4321.55 | 17.6 | 80.03 | 0.332 |
| **Residual** | 159 | 11650.28 | 47.4 | 73.27 | |
| **Total** | 239 | 24560.86 | | 102.77 | |
| SE = 8.56 | CV = 13.17 % | Adj. $R^2$ = 29 % | | | |

df, degrees of freedom; SS, sum of squares; SS (%), SS as percentage to the total; MS, mean squares; p, probability; SE, standard error; Adj. $R^2$, adjusted $R^2$ (%); CV, coefficient of variation (%).

### 3.4.3 Phytic acid and mineral interactions

We found some interesting associations between phytic acid and other mineral elements in white flour samples. Phytic acid had a strong and positive relationship with P (r=0.878, p<0.001), Mg (r=0.680, p<0.001), Fe (r=0.625, p<0.001), and also positive but weaker relationships with K (r=0.393, p<0.001), Zn (r=0.315, p<0.001) and Ca (r=0.217, p<0.001), whereas for Cu (r= - 0.376, p<0.001), there was a negative relationship. This correlation analysis was done including all the data

available, even the points substituted with the half-LOD, it is worth noting that if these values are eliminated the correlations were very similar, Figure 3-6.

Additionally, Table 3-13 shows with more detail the associations between phytic acid and four other mineral elements (Ca, Fe, P and Zn) according to the site and the harvest year.

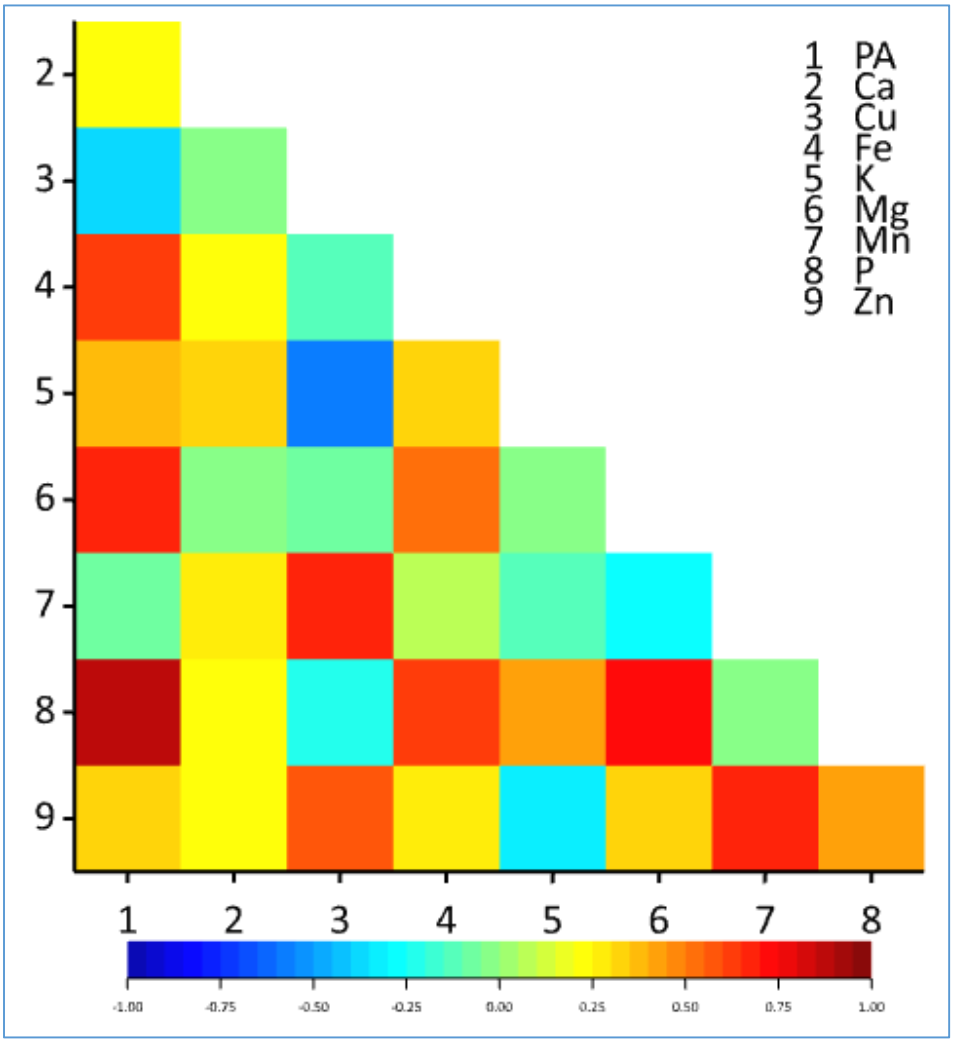



Figure 3-6 Relationship between the concentrations of phytic acid and other 8 mineral elements in white flour of a panel of 24 genotypes grown at two sites in the UK. Data of two harvest years. Colour corresponds to the strength and direction of the correlation from strongly negative (dark blue) to strongly positive (dark red).

There were highly significant differences ($p<0.001$) in P, Ca, Fe and Zn between the sites and harvest years and among the genotypes, Table 3-12.

Calcium concentrations were significantly higher ($p<0.001$, LSD 1 %=11.02) at RRes (222.7 mg $kg^{-1}$, n=114) than at UoN (207.7 mg $kg^{-1}$, n=129) in white flour, and equally in wholegrain flour ($p<0.001$, LSD 1 %=26.69), genotypes grown at RRes had significantly more Ca concentration (489.6 mg $kg^{-1}$) than when grown at UoN (420.03 mg $kg^{-1}$).

Phosphorus concentrations were significantly higher ($p<0.001$, LSD 1 %=66.5) at UoN (1308 mg $kg^{-1}$, n=129) than at RRes (1207 mg $kg^{-1}$, n=114) in white flour, and equally in wholegrain flour ($p<0.001$, LSD 1 %=159.4), genotypes grown at UoN had significantly more P concentration (4239 mg $kg^{-1}$) than when grown at RRes (3899 mg $kg^{-1}$).

Table 3-12 One-way ANOVA studying P, Ca, Fe and Zn concentrations between the sites and harvest years and among the genotypes.

| | **Site** | | **Harvest year** | | **Genotype** | |
|---|---|---|---|---|---|---|
| | **WF** | **WGF** | **WF** | **WGF** | **WF** | **WGF** |
| **P** | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Ca** | <0.001 | <0.001 | <0.001 | 0.004 | <0.001 | <0.001 |
| **Fe** | 0.051 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Zn** | <0.001 | 0.09 | 0.003 | <0.001 | <0.001 | <0.001 |

A simple linear regression analysis was carried out to explore if the phosphorus concentrations could explain the phytic acid concentrations in white flour and in wholegrain flour. For white flour (WF) the regression was significant at RRes site

(p<0.001, SE= 115) and at UoN site (p<0.001, SE=199). Figure 3-7 shows the equations for each site and the corresponding adjusted R-squared.

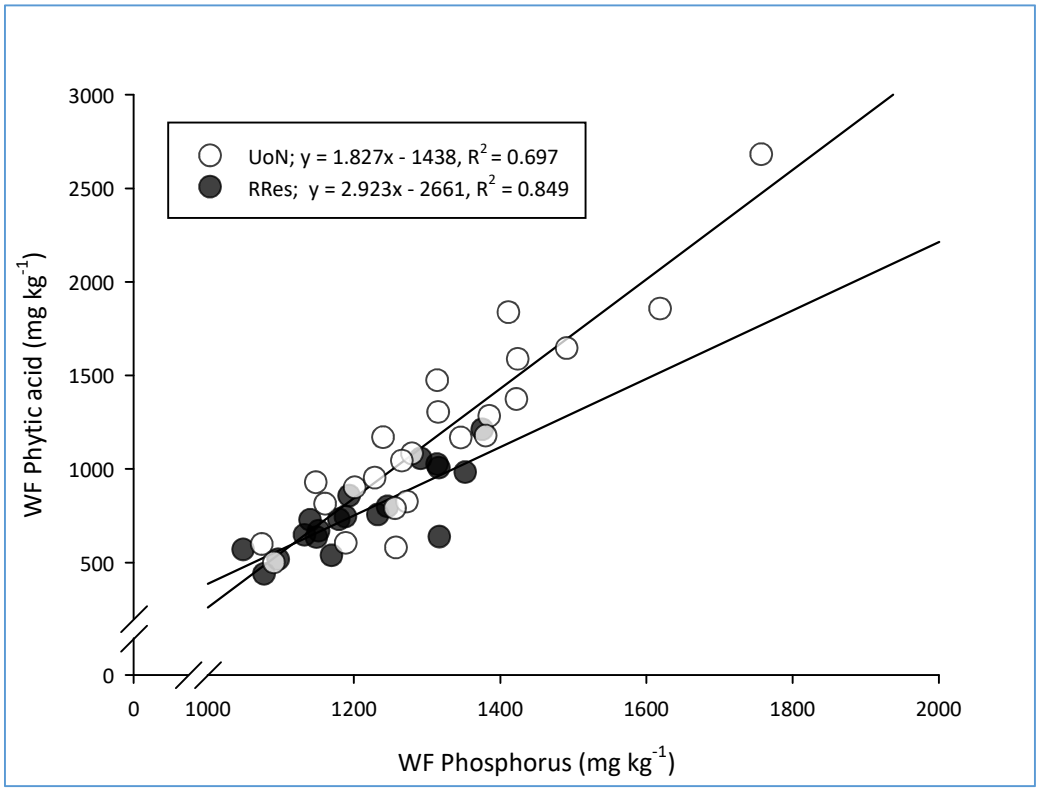



Figure 3-7 Linear relationship between phosphorus and phytic acid concentration in white flour (WF) of 24 genotypes grown at two sites in the UK. Data points correspond to mean values from two harvest years. RRes , Rothamsted Research, UoN , University of Nottingham, $R^2$ , adjusted $R^2$.

Similarly, a correlation analysis was performed in WGF samples, in this case phytic acid had a positive relationship with P (r=0.495, p<0.001), Mg (r=0.263, p<0.001), Zn (r=0.173, p=0.007), Fe (r=0.150, p=0.020), Figure 3-8.

Table 3-12 shows the probabilities of the differences between sites and harvest years and among the genotypes for these minerals. Additionally, Table 3-13 shows

with more detail the associations between phytic acid and four other mineral elements (Ca, Fe, P and Zn) according to the site and the harvest year.

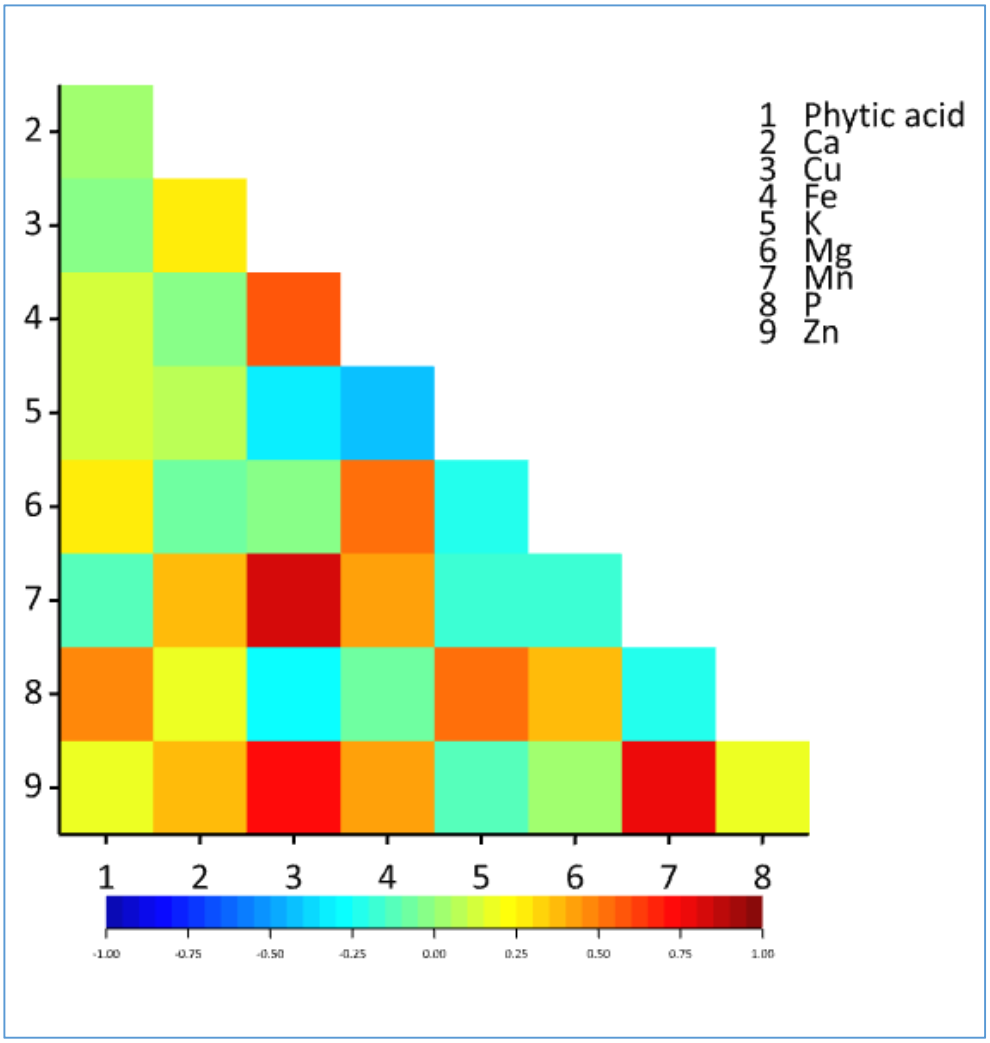



Figure 3-8 Relationship between the concentrations of phytic acid and other 8 mineral elements in wholegrain flour of a panel of 24 genotypes grown at two sites in the UK. Data of two harvest years. Colour corresponds to the strength and direction of the correlation from strongly negative (dark blue) to strongly positive (dark red).

Table 3-13 Correlation coefficients of phytic acid and four mineral elements (Ca, Fe, P and Zn) in a panel of 24 genotypes grown in the UK at two different sites in two harvest years.

| | Phytic acid, Nottingham - HY1 | | | | Phytic acid, Nottingham - HY2 | | | |
|---|---|---|---|---|---|---|---|---|
| **Mineral** | **WF** | | **WGF** | | **WF** | | **WGF** | |
| | **Correlation coefficient** | **p** | **Correlation coefficient** | **p** | **Correlation coefficient** | **p** | **Correlation coefficient** | **p** |
| **Ca** | 0.156 | 0.20 | 0.099 | 0.42 | -0.211 | 0.12 | 0.346 | 0.009 |
| **Fe** | 0.558 | < 0.001 | -0.023 | 0.85 | 0.670 | < 0.001 | 0.345 | 0.009 |
| **P** | 0.828 | < 0.001 | 0.424 | < 0.001 | 0.846 | < 0.001 | 0.647 | < 0.001 |
| **Zn** | 0.552 | < 0.001 | 0.202 | 0.09 | 0.560 | < 0.001 | 0.547 | < 0.001 |
| | **Phytic acid, Rothamsted - HY1** | | | | **Phytic acid, Rothamsted - HY2** | | | |
| **Mineral** | **WF** | | **WGF** | | **WF** | | **WGF** | |
| | **Correlation coefficient** | **p** | **Correlation coefficient** | **p** | **Correlation coefficient** | **p** | **Correlation coefficient** | **p** |
| **Ca** | 0.269 | 0.051 | -0.065 | 0.63 | -0.196 | 0.14 | -0.030 | 0.82 |
| **Fe** | 0.215 | 0.123 | 0.331 | 0.01 | 0.620 | < 0.001 | 0.451 | < 0.001 |
| **P** | 0.703 | < 0.001 | 0.398 | 0.002 | 0.761 | < 0.001 | 0.603 | < 0.001 |
| **Zn** | 0.655 | < 0.001 | 0.245 | 0.07 | 0.633 | < 0.001 | 0.404 | 0.002 |

HY1, harvest year 1; HY2, harvest year 2; WF, white flour; WGF, wholegrain flour; p, p value.

Likewise, for wholegrain flour (WGF) the regression was significant at RRes (p=0.002, SE=693) and at UoN (p<0.001, SE=712). Figure 3-9 shows the equations for each of the sites and the corresponding adjusted R-squared.

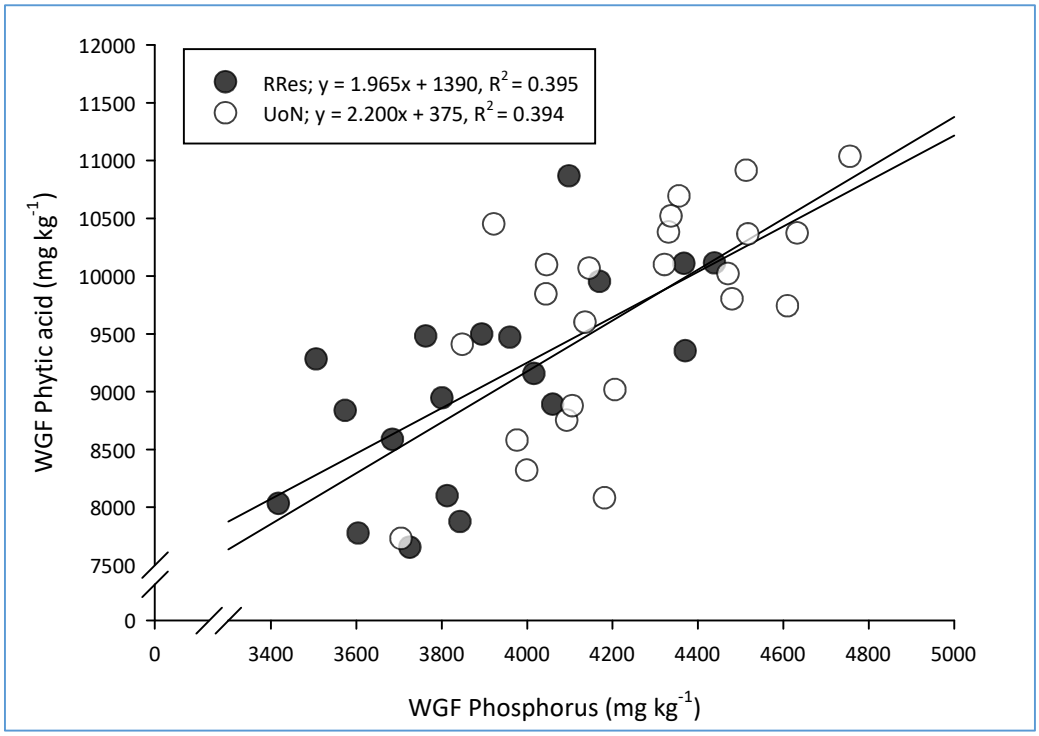



Figure 3-9 Linear relationship between phosphorus and phytic acid concentration in wholegrain flour (WGF) of 24 genotypes grown at two sites in the UK. Data points correspond to mean values from two harvest years. RRes, Rothamsted Research; UoN, University of Nottingham; $R^2$, adjusted $R^2$.

### 3.4.4 PA concentrations in the four different levels of Fe-Zn in white and wholegrain flour samples

In WF samples, the level with the highest PA concentration was level 2 (LFe-HZn, in both sites, 902.5 and 1316.8 mg $kg^{-1}$ at RRes and UoN respectively. The lowest PA concentration was level 3 (HFe-LZn) at RRes site, 650.2 mg $kg^{-1}$ and at UoN was level 4 (LFe-LZn), 948.1 mg $kg^{-1}$. One-way ANOVA showed levels were significant

(p=0.017, LSD 5 %= 258.4) for the PA concentration in white flour. Figure 3-10 shows the significant differences between the levels using the pooled means of both sites.

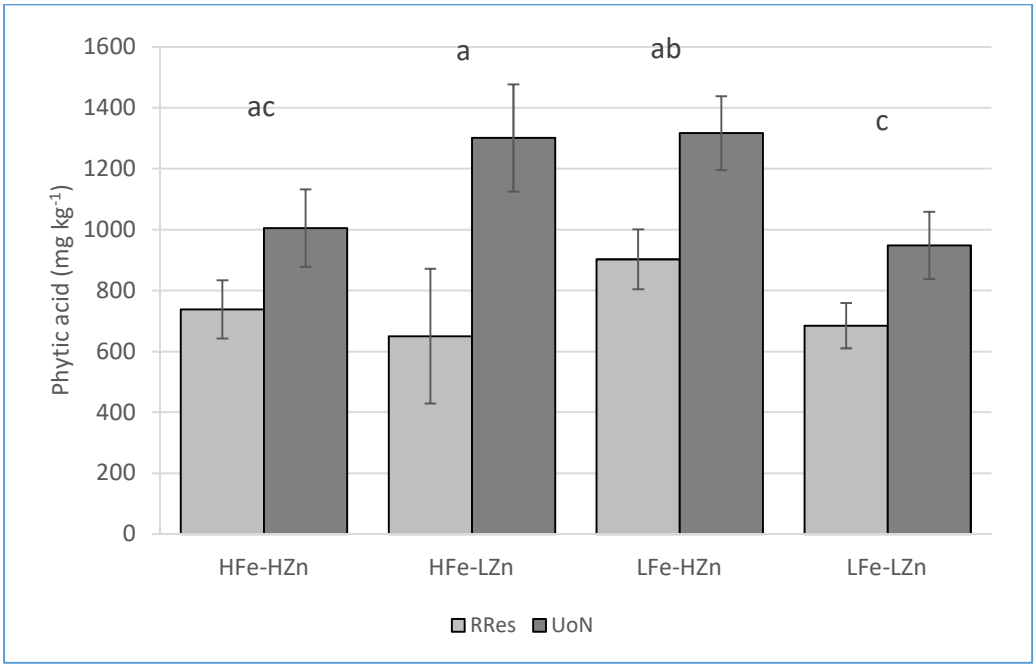



Figure 3-10 Phytic acid concentration in white flour according to Fe-Zn levels. Panel of 24 genotypes grown in two sites in the UK. Means followed by a common letter are not significantly different by One-way ANOVA at the 5 % level of significance. Means ± SE bars. Two harvest year data. Data are in mg $kg^{-1}$. SE, standard error; L, low; H, high.

In wholegrain flour samples, the level with the highest PA concentration was level 1 (HFe-HZn), in both sites, 9815 mg $kg^{-1}$ at RRes and 10347 mg $kg^{-1}$ at UoN. The lowest PA concentration was in level 4 (LFe-LZn) also at both sites, 8511 mg $kg^{-1}$ at RRes and 8645 mg $kg^{-1}$ at UoN, see Figure 3-11.

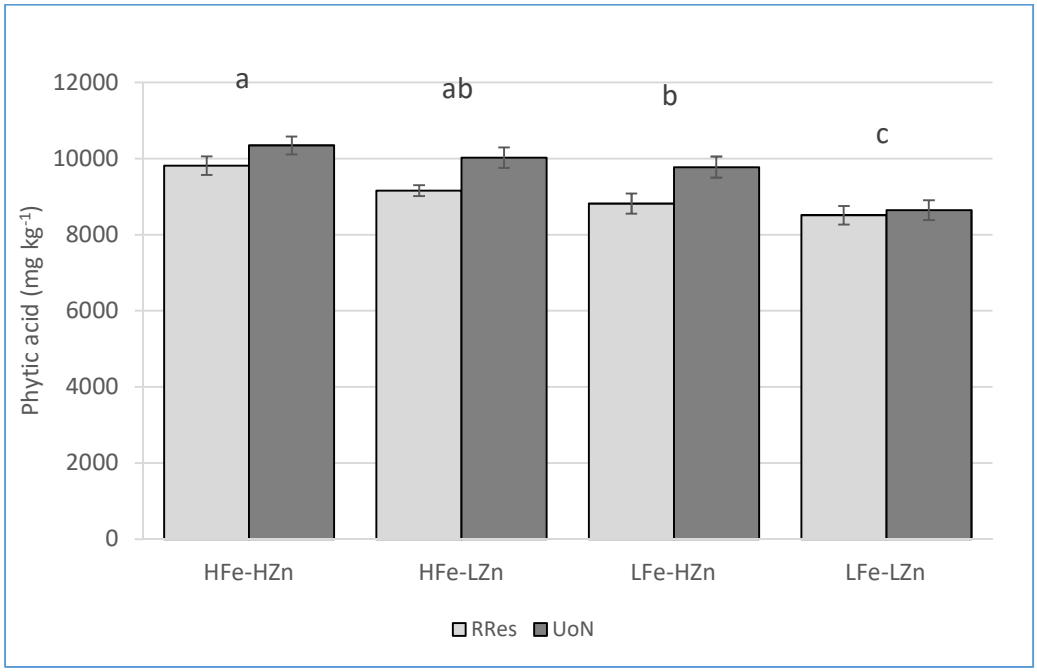



Figure 3-11 Phytic acid concentration in wholegrain flour according to Fe-Zn levels. Panel of 24 genotypes grown in two sites in the UK. Means followed by a common letter are not significantly different by One-way ANOVA at the 5 % level of significance. Means ± SE bars. Two harvest year data. Data are in mg $kg^{-1}$. SE, standard error; L, low; H, high.

The PA differences between the four levels were evaluated between sites and harvest years independently using one-way ANOVA. In white flour, only level 2 had significant differences in PA concentrations between sites and all levels showed highly significant differences between the harvest years. On the other hand, for wholegrain flour there were significant differences between sites only in level 2, whereas between the years there were not significant differences, Table 3-14 shows the significance values for each combination.

Table 3-14 One-way ANOVA studying the performance of four levels of genotypes with different Fe and Zn concentrations in white flour (WF) and wholegrain flour (WGF) between the sites and harvest years. Each group consists of 6 genotypes and data are 3 replicate plots per genotype at each site and harvest year.

| | PA concentration | | | |
|---|---|---|---|---|
| **Levels** | **Site** | | **Harvest year** | |
| | **WF** | **WGF** | **WF** | **WGF** |
| **1-High_Fe - High_Zn** | 0.097 | 0.121 | <0.001 | 0.343 |
| **2-Low_Fe - High_Zn** | 0.010 | 0.014 | <0.001 | 0.256 |
| **3-High_Fe-Low_Zn** | 0.077 | 0.106 | <0.001 | 0.090 |
| **4-Low_Fe-Low_Zn** | 0.052 | 0.707 | <0.001 | 0.283 |

In WGF there was significantly more PA, P and phytic acid-P/total P % in the High-Fe genotypes (levels 1 and 3) than in the Low-Fe genotypes (levels 2 and 4), this did not occur in WF. Additionally, in WGF there was significantly more PA, P and phytic acid-P/total P % in the High-Zn genotypes (levels 1 and 2) than in the Low-Zn genotypes (levels 3 and 4), in WF this only occurred for P concentrations, Table 3-15.

Table 3-15 Comparison of concentrations of PA, P and Phytic acid-P/total P % between genotypes sorted in two categories of Fe and Zn (high and low). Data are mean values in mg $kg^{-1}$, except when indicated as percentage (%).

| | **High-Fe** | **Low-Fe** | **p value** | **LSD** |
|---|---|---|---|---|
| **PA (WGF)** | 10013 | 8932 | <0.001 | 517.9 |
| **Phytic acid-P/total P % (WGF)** | 67.7 % | 63.2 % | <0.001 | 3.36 |
| **P (WGF)** | 4207 | 3988 | <0.001 | 168 |
| | High-Zn | Low-Zn | p value | LSD |
| **PA (WGF)** | 9689 | 8920 | <0.001 | 533.2 |
| **P (WGF)** | 4187 | 3919 | <0.001 | 166 |
| **P (WF)** | 1287 | 1224 | 0.017 | 68.9 |

High-Fe, n=99; Low-Fe, n=144; High-Zn, n=144; Low-Zn, n=99; WF, white flour; WGF, wholegrain flour.

### 3.4.5 Phytic acid-mineral ratios in white flour and wholegrain flour wheat

Phytic acid ratios were calculated for three minerals (Ca, Fe and Zn). Ratios were expressed as millimolar ratios and were calculated dividing the mmol of PA by the mmol of the mineral element. The following molecular weights were used for these calculations: PA=660.04, Ca=40.08, Fe=55.85 and Zn=65.4 g $mol^{-1}$.

PAxCa:Zn ratio has been reported to be a better approximation of the Zn bioavailability, therefore it was also included in this analysis. The calculation was made by obtaining the Ca:Zn ratio and multiplying it by the mmol of PA. In the following paragraphs the results of the statistical analysis are shown, first for white flour samples and afterwards for wholegrain flour.

Table 3-16 presents a summary of the lowest and highest PA ratios and the county of origin of the corresponding genotypes.

Table 3-16 Summary of phytic acid to Ca, Fe and Zn ratios and the corresponding country of origin. Panel of 24 genotypes grown in two sites in the UK in two harvest years. Values for each ratio are in parenthesis.

| White flour | | | | |
|---|---|---|---|---|
| **Ratio** | **Lowest** | **Origin** | **Highest** | **Origin** |
| **PA:Ca** | PxW264-50 (0.12) | Canary Islands | PxW546-25 (0.62) | Spain |
| **PA:Fe** | PxW299-87 (3.8) | Turkey | PxW546-25 (11.3) | Spain |
| **PA:Zn** | PxW811-30 (5) | Tunisia | PxW546-25 (20) | Spain |
| **PA x Ca:Zn** | PxW811-30 (23) | Tunisia | PxW546-25 (120.8) | Spain |
| **Wholegrain flour** | | | | |
| **Ratio** | **Lowest** | **Origin** | **Highest** | **Origin** |
| **PA:Ca** | PxW264-50 (1.0) | Canary Islands | PxW7-76 (1.8) | Australia |
| **PA:Fe** | PxW7-60 (18.8) | Australia | PxW291-39 (30.9) | Cyprus |
| **PA:Zn** | PxW396-56 (23.2) | Portugal | PxW398-18 (41.8) | Palestine |
| **PA x Ca:Zn** | PxW811-30 (249.5) | Tunisia | PxW264-50 (467.4) | Canary Islands |

Cell colours represent the Fe-Zn level: High Fe-High Zn (red); Low Fe-High Zn (blue); High Fe-Low Zn (black); Low Fe-Low Zn (green).

#### *3.4.5.1 White flour*

One-way ANOVA was carried out to look for differences between sites and years in each of the ratios calculated. There were highly significant differences between the sites in white flour for all the ratios: PA:Ca ($p<0.001$), PA:Fe ($p<0.001$), PA:Zn ($p<0.001$) and PAxCa:Zn ($p<0.001$). UoN site had the highest mean for all four calculations. The summary statistics can be found in Table 3-17.

Table 3-17 Phytic acid: Ca, Fe, Zn millimolar ratio in white flour of a panel of 24 genotypes of wheat grown in the UK in two sites. Data are values averaged across two harvest years.

| Ratio | n | Mean | SD | Median | Minimum | Maximum |
|---|---|---|---|---|---|---|
| PA:Ca RRes | 110 | 0.20 | 0.14 | 0.19 | 0.04 | 0.63 |
| PA:Ca UoN | 125 | 0.33 | 0.21 | 0.31 | 0.03 | 0.88 |
| PA:Fe RRes | 110 | 4.75 | 3.06 | 4.08 | 1.15 | 12.41 |
| PA:Fe UoN | 124 | 6.28 | 3.44 | 5.78 | 1.01 | 14.54 |
| PA:Zn RRes | 110 | 5.84 | 4.02 | 4.45 | 1.17 | 15.66 |
| PA:Zn UoN | 125 | 11.02 | 5.91 | 10.69 | 1.20 | 26.27 |
| PAxCa:Zn RRes | 110 | 33.51 | 24.97 | 22.72 | 5.57 | 98.70 |
| PAxCa:Zn UoN | 125 | 58.46 | 36.37 | 51.72 | 6.82 | 182.28 |

n, number of values; SD, standard deviation; RRes, Rothamsted Research; UoN, University of Nottingham.

In terms of the harvest year, all the ratios had highly significant differences. PA:Ca ratios with means of 0.3951 ± 0.1608 in harvest year 1 and 0.1288 ± 0.0954 in harvest year 2, $p<0.001$; PA:Fe with means of 7.884 ± 2.791 in harvest year 1 and 3.031 ± 1.654 in harvest year 2, $p<0.001$; PA:Zn with mean values of 12.539 ± 4.658 in harvest year 1 and 4.331 ± 3.119 in harvest year 2, $p<0.001$; and for PAxCa:Zn with means of 70.31 ± 29.21 in harvest year 1 and 21.38 ± 14.85 in harvest year 2, $p<0.001$ (values are means ± standard deviation).

One-way ANOVA showed significant differences between genotypes: PA:Ca, PA:Zn, PAxCa:Zn ($p<0.001$) and PA:Fe ($p=0.010$). The range for PA:Ca ratios was 0.12 ± 0.10 to 0.68 ± 0.13 (mean ± SD), which corresponded to genotypes PxW264 – 50 and PxW546 – 25, respectively. For PA:Fe ratios, the range found was 3.79 ± 2.76 to 11.31 ± 3.11, these corresponded to genotypes PxW299 – 87 and PxW546

– 25, respectively. For PA:Zn ratios the range was 4.99 ± 3.65 to 19.99 ± 1.11, and corresponded to genotypes PxW811 – 30 and PxW546 – 25. Finally for PAxCa:Zn ratios the range was 22.99 ± 16.52 to 120.81 ± 3.49 corresponding to genotypes PxW811 – 30 and PxW546 – 25, respectively. In Figure 3-12 and Figure 3-13 are depicted the mean ratios of Fe, Zn and PAxCa:Zn by genotype. Table 3-20 shows a summary of the significances for each ratio.

All the genotypes had values above the critical value for good bioavailability of Fe (PA:Fe < 1). For Zn, with the exception of two genotypes (PxW223 – 80 and PxW546 – 25) all had ratios lower than the recommended critical value (PA:Zn < 15). For PAxCa:Zn ratios, none of the genotypes were above the recommended value for good Zn bioavailability (PAxCa:Zn < 200).

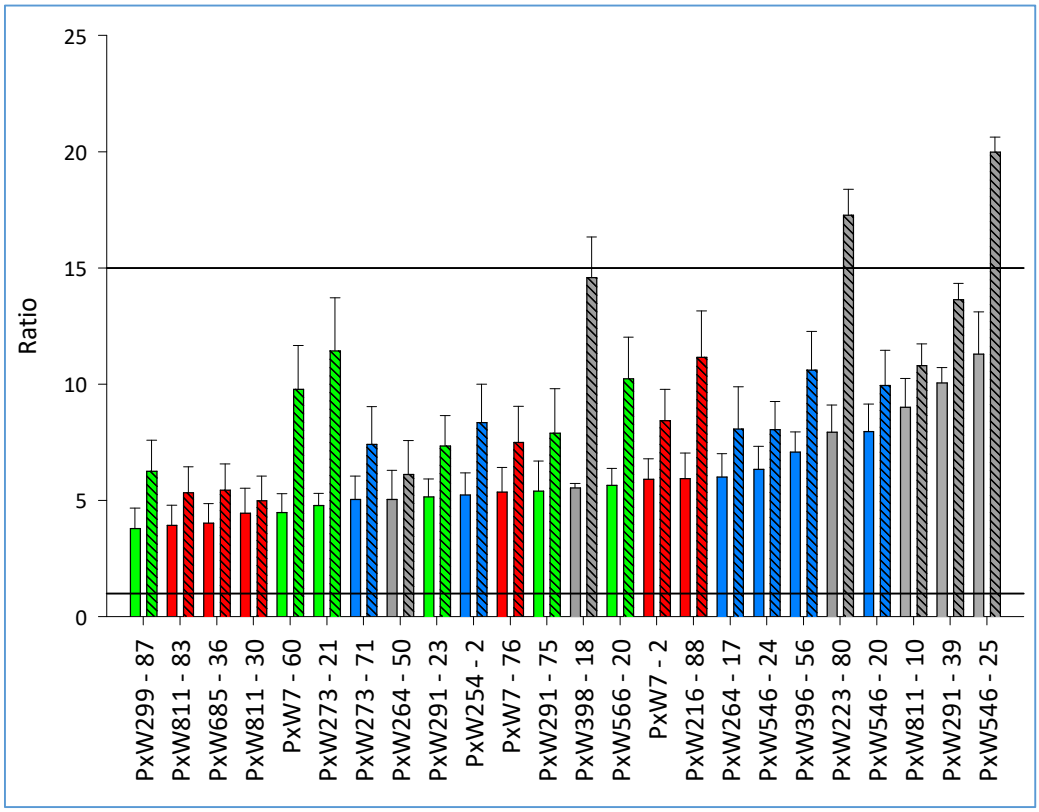



Figure 3-12 Phytic acid to Fe and Zn ratios in white flour of a panel of 24 genotypes of wheat grown in the UK in two sites during two harvest years. Mean ± SE bars. Solid bars are PA:Fe ratios, patterned bars are PA:Zn ratios. Genotypes are sorted according to PA:Fe values from lowest to highest. Colours represent the Fe-Zn levels: Red= HighFe-HighZn; Blue= LowFe-HighZn; Grey= HighFe-LowZn, Green= LowFe-LowZn. The reference lines are the critical values for Fe (y=1) and Zn (y=15) bioavailability.

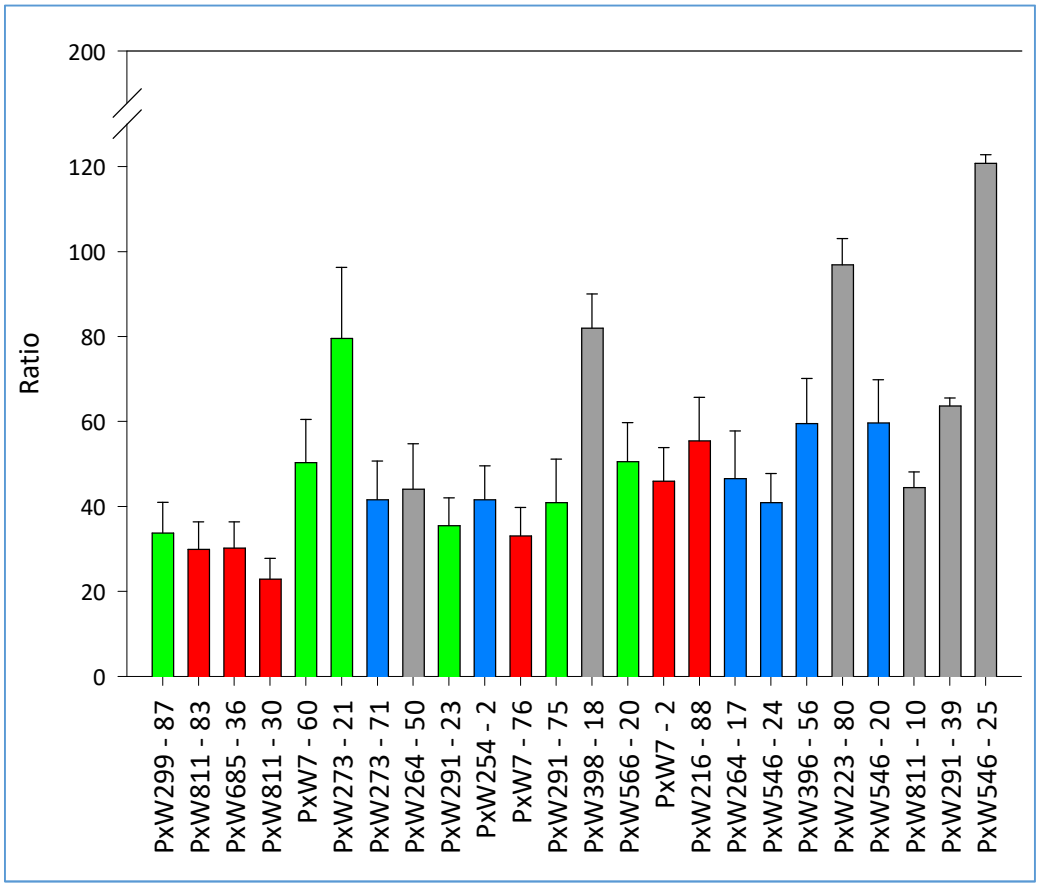



Figure 3-13 Phytic acid x Ca:Zn ratio in white flour of a panel of 24 genotypes of wheat grown in the UK in two sites during two harvest years. Genotypes are ranked as in Figure 3-12 for comparison purposes. Colours represent the Fe-Zn levels: Red= HighFe-HighZn; Blue= LowFe-HighZn; Grey= HighFe-LowZn, Green= LowFe-LowZn. The reference line at y=200 represents the critical value for good Zn bioavailability.

Genotype accounted for 17-27 % (of sum of squares-SS) of the variation in PA ratios in WF while the environment (combination of site-year) accounted for a greater proportion of the variation, 46-55 % while the G x E interaction accounted for 8-12 % and the residual term was associated with 11-22 % of the variance, Table 3-18.

Table 3-18 Variation in PA to Ca, Fe, Zn and Ca:Zn ratios due to G, E, G x E and residual variance component factors in a panel of 24 genotypes derived from Watkins landraces grown at two sites in the UK during two harvest years.

| | G | p | E | p | G x E | p | Residual |
|---|---|---|---|---|---|---|---|
| **White flour (SS %)** | | | | | | | |
| **PA:Ca** | 27.1 | <0.001 | 51.4 | <0.001 | 8.1 | 0.005 | 13.3 |
| **PA:Fe** | 17.2 | <0.001 | 49.1 | <0.001 | 11.6 | 0.033 | 22.1 |
| **PA:Zn** | 27.1 | <0.001 | 51.4 | <0.001 | 8.1 | 0.005 | 13.3 |
| **PA x Ca:Zn** | 24.9 | <0.001 | 55.3 | <0.001 | 9.0 | <0.001 | 10.8 |
| **Wholegrain flour (SS %)** | | | | | | | |
| **PA:Ca** | 32.3 | <0.001 | 18.2 | <0.001 | 14.7 | 0.147 | 34.7 |
| **PA:Fe** | 13.9 | <0.001 | 39.2 | <0.001 | 11.2 | 0.622 | 35.7 |
| **PA:Zn** | 14.4 | <0.001 | 51.5 | <0.001 | 9.1 | 0.369 | 25.0 |
| **PA x Ca:Zn** | 31.3 | <0.001 | 23.9 | <0.001 | 15.2 | 0.024 | 29.5 |

PA, phytic acid; SS %, percentage of variation of the sum of squares; G, genotype; E, environment (combination of site-year); p, probability value.

As a summary, for white flour, all genotypes had PAxCa:Zn ratios <200 in white flour, indicating a good zinc bioavailability. All genotypes, except PxW223 – 80 and PxW546 – 25, had PA:Zn ratios <15 which indicates Zn bioavailability would not be compromised by the phytate levels and indicating that approximately, 35 % of the zinc would be absorbed. On the contrary, because all the genotypes had >1 PA:Fe ratios, it is likely the Fe bioavailability would be compromised. Ten out of 24 genotypes had a PA:Ca ratio <0.24, PxW264 – 50, PxW299 – 87, PxW811 – 83, PxW291 – 75, PxW811 – 30, PxW685 – 36, PxW273 – 71, PxW264 – 17, PxW273 – 21, PxW291 – 23, which means they would have good Ca bioavailability. These 10 genotypes would comply with having good bioavailability of Ca and Zn but it is likely that the Fe bioavailability would be affected.

### *3.4.5.2 Wholegrain flour*

For wholegrain flour samples, one-way ANOVA was carried out to look for differences between sites and harvest years independently. There were highly significant differences between the sites in all four calculated ratios: PA:Ca ($p<0.001$), PA:Fe ($p<0.001$), PA:Zn ($p<0.001$) and PAxCa:Zn ($p<0.001$). UoN site had the highest means for all four phytate to mineral ratios, see Table 3-19.

Table 3-19 Phytic acid (PA): Ca, Fe, Zn millimolar ratio in wholegrain flour of a panel of 24 genotypes of wheat grown in the UK in two sites. Data are values averaged across two harvest years.

| Ratios | n | Mean | SD | Median | Minimum | Maximum |
|---|---|---|---|---|---|---|
| PA:Ca RRes | 114 | 1.16 | 0.29 | 1.14 | 0.57 | 1.92 |
| PA:Ca UoN | 126 | 1.42 | 0.32 | 1.38 | 0.90 | 2.86 |
| PA:Fe RRes | 114 | 21.59 | 5.77 | 20.42 | 12.51 | 40.04 |
| PA:Fe UoN | 126 | 25.11 | 6.72 | 23.80 | 12.76 | 43.47 |
| PA:Zn RRes | 114 | 23.18 | 4.82 | 22.73 | 11.12 | 37.42 |
| PA:Zn UoN | 126 | 36.86 | 7.77 | 37.35 | 16.79 | 56.58 |
| PAxCa:Zn RRes | 114 | 279.65 | 60.06 | 276.02 | 142.28 | 469.03 |
| PAxCa:Zn UoN | 126 | 389.90 | 119.45 | 375.76 | 178.53 | 786.57 |

n, number of values; SD, standard deviation; RRes, Rothamsted Research; UoN, University of Nottingham.

In terms of harvest year, significant differences were found only for PA:Fe ratios, which had means of 27.02 ± 6.488 in harvest year 1 and 19.42 ± 3.510 in harvest year 2, $p<0.001$. The other three ratios did not have any differences on both years; PA:Ca ratios, means of 1.295 ± 0.3832 in harvest year 1 and 1.301 ± 0.2611 in harvest year 2, $p=0.892$; PA:Zn, with mean values of 29.76 ± 9.563 in harvest year

1 and 31.05 ± 9.334 in harvest year 2, p=0.293 and PAxCa:Zn with means of 342.5 ± 118.2 in harvest year 1 and 331.9 ± 101.3 in harvest year 2, p=0.459 (values are means ± standard deviation).

There were significant differences between genotypes in PA:Ca and PAxCa:Zn ($p<0.001$) but not for PA:Zn, p=0.051 or PA:Fe, p=0.066. The range for PA:Ca ratio was 1.0 ± 0.16 to 1.81 ± 0.39, corresponding to genotypes PxW264 – 50 and PxW7 – 76, respectively. For PAxCa:Zn ratios the range was 249.51 ± 49.47 to 467.39 ± 144.79, corresponding to genotypes PxW811 – 30 and PxW564 – 50 respectively. The range in PA:Fe ratios was 18.81 ± 5.96 to 30.86 ± 8.12 and corresponded to genotypes PxW7 – 60 and PxW291 – 39. In PA:Zn ratios the range was 23.24 ± 6.16 to 41.78 ± 6.24 which corresponded to genotypes PxW396 – 56 and PxW398 – 18, (values are means ± SD). In wholegrain flour samples all genotypes exceeded critical values. See Figure 3-14 and Figure 3-15. Table 3-20 shows a summary of the significances for each ratio.

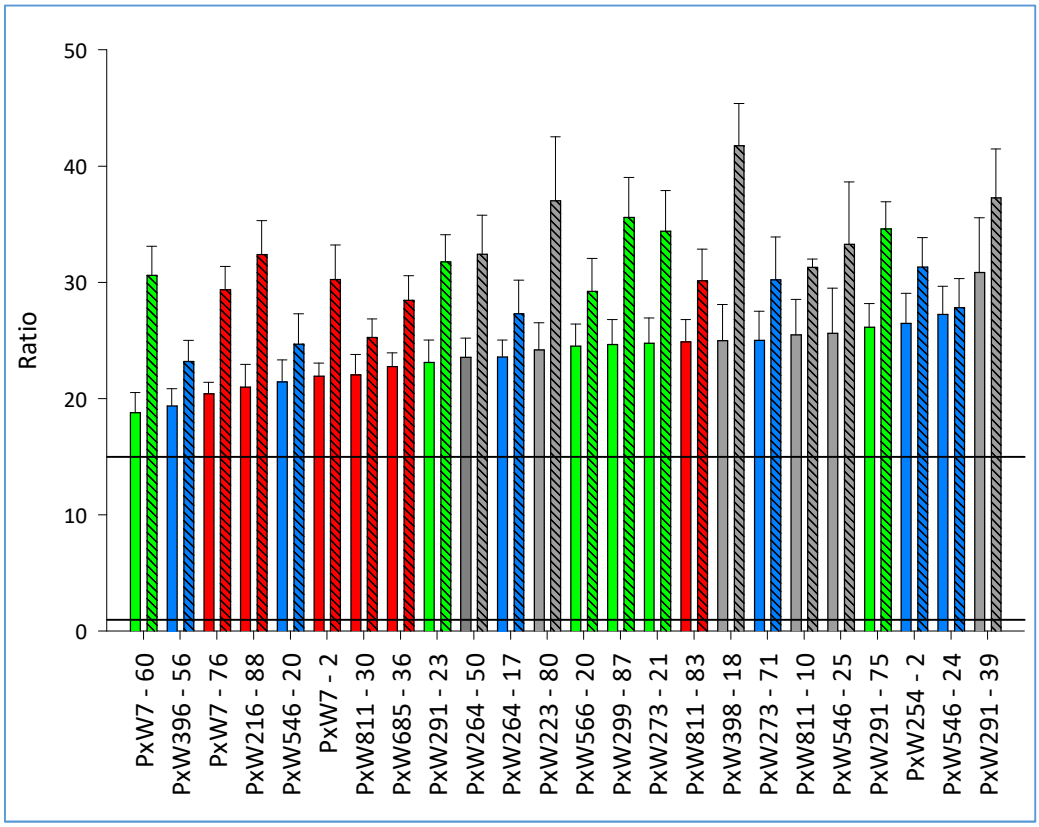



Figure 3-14 Phytic acid to Fe and Zn ratios in wholegrain flour of a panel of 24 genotypes of wheat grown in the UK in two sites during two harvest years. Mean ± SE bars. Solid bars are PA:Fe ratios, patterned bars are PA:Zn ratios. Genotypes are sorted according to PA:Fe values from lowest to highest. Colours represent the Fe-Zn levels: Red= HighFe-HighZn; Blue= LowFe-HighZn; Grey= HighFe-LowZn, Green= LowFe-LowZn. The reference lines are the critical values for Fe (y=1) and Zn (y=15) bioavailability.

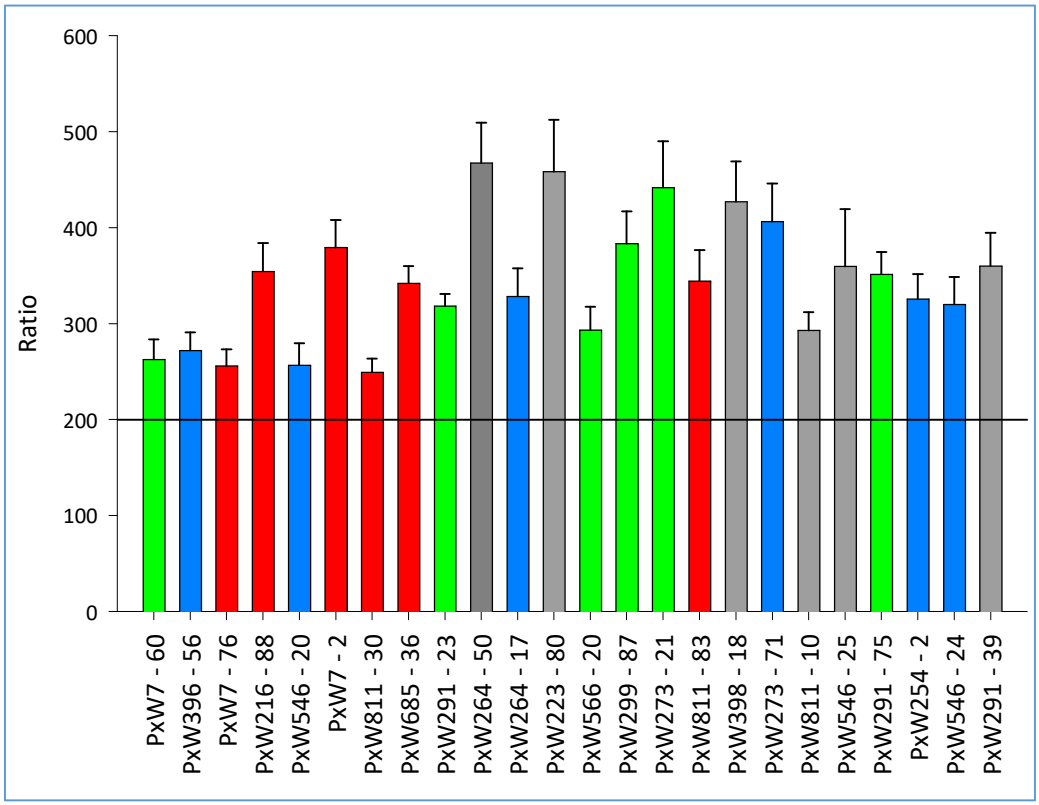



Figure 3-15 Phytic acid x Ca:Zn ratio in wholegrain flour of a panel of 24 genotypes of wheat grown in the UK in two sites during two harvest years. Genotypes are ranked as in Figure 3-14 for comparison purposes. Colours represent the Fe-Zn levels: Red= HighFe-HighZn; Blue= LowFe-HighZn; Grey= HighFe-LowZn, Green= LowFe-LowZn. The reference lines are the critical values for Fe (y=1) and Zn (y=15) bioavailability.

Table 3-20 One-way ANOVA studying the PA:Fe, PA:Zn, PA:Ca and PAxCa:Zn ratios in white flour (WF) and wholegrain flour (WGF) of a panel of 24 genotypes grown in two sites in the UK in two harvest years.

| Ratio | Site | | Harvest year | | Genotype | |
|---|---|---|---|---|---|---|
| | WF | WGF | WF | WGF | WF | WGF |
| **PA:Ca** | <0.001 | <0.001 | <0.001 | 0.892 | <0.001 | <0.001 |
| **PA:Fe** | <0.001 | <0.001 | <0.001 | <0.001 | 0.01 | 0.066 |
| **PA:Zn** | <0.001 | <0.001 | <0.001 | 0.293 | <0.001 | 0.051 |
| **PA x Ca:Zn** | <0.001 | <0.001 | <0.001 | 0.459 | <0.001 | <0.001 |

Differences between the levels for each site and harvest year were analysed for each ratio, Table 3-21. In white flour samples, for PA:Ca ratios there were significant differences ($p<0.01$) between the levels and site for level 2 in WF and 1, 2 and 3 in WGF and between levels and year for all levels only in WF.

For PA:Fe, there were significant differences ($p<0.01$) between levels and sites for level 2 in WGF and between levels and years for all levels in both WF and WGF.

Table 3-21 One-way ANOVA studying the PA ratios of four levels of genotypes with different Fe and Zn concentrations in white flour (WF) and wholegrain flour (WGF) between the sites and harvest years. Each group consists of 6 genotypes and data are 3 replicate plots per genotype at each site and harvest year.

| | PA:Ca | | | | PA:Fe | | | |
|---|---|---|---|---|---|---|---|---|
| **Levels** | **Site** | | **Harvest year** | | **Site** | | **Harvest year** | |
| | **WF** | **WGF** | **WF** | **WGF** | **WF** | **WGF** | **WF** | **WGF** |
| **1-High_Fe - High_Zn** | 0.013 | <0.001 | <0.001 | 0.778 | 0.013 | 0.046 | <0.001 | <0.001 |
| **2-Low_Fe - High_Zn** | 0.001 | <0.001 | <0.001 | 0.44 | 0.138 | 0.008 | <0.001 | <0.001 |
| **3-High_Fe-Low_Zn** | 0.018 | 0.004 | <0.001 | 0.024 | 0.469 | 0.051 | <0.001 | 0.008 |
| **4-Low_Fe-Low_Zn** | 0.017 | 0.014 | <0.001 | 0.823 | 0.161 | 0.084 | <0.001 | <0.001 |
| | **PA:Zn** | | | | **PA x Ca:Zn** | | | |
| **Levels** | **Site** | | **Year** | | **Site** | | **Year** | |
| | **WF** | **WGF** | **WF** | **WGF** | **WF** | **WGF** | **WF** | **WGF** |
| **1-High_Fe - High_Zn** | 0.001 | <0.001 | <0.001 | 0.029 | 0.015 | <0.001 | <0.001 | 0.385 |
| **2-Low_Fe - High_Zn** | <0.001 | <0.001 | <0.001 | 0.595 | <0.001 | <0.001 | <0.001 | 0.377 |
| **3-High_Fe-Low_Zn** | 0.006 | <0.001 | <0.001 | 0.874 | 0.06 | 0.08 | <0.001 | 0.151 |
| **4-Low_Fe-Low_Zn** | <0.001 | <0.001 | <0.001 | 0.894 | 0.006 | <0.001 | <0.001 | 0.438 |

For PA:Zn, there were significant differences ($p<0.01$) between levels and sites for all levels in WF and WGF and between levels and years for all levels but only in WF. Lastly, for PAxCa:Zn ratios, there were significant differences ($p<0.01$)

between levels and sites in levels 2 and 4 in WF and 1, 2 and 4 in WGF and between levels and years for all levels but only in WF.

Genotype accounted for 14.4-31.3 % (of sum of squares-SS) of the variation in PA ratios in WGF. The environment (combination of site-year) accounted for 18.2-51.5 % while the G x E interaction accounted for 9-15 % and the residual term was associated with 25-36 % of the variance, Table 3-18.

The variance in all the ratios seemed more driven by the environmental factor in WF and only PA:Fe and PA:Zn in the WGF. The variation in PA:Ca and PAxCa:Zn was more driven by the genotype in WGF. Only for PAxCa:Zn in WF the interaction G x E was highly significant ($p<0.001$) and only for PAxCa:Zn the interaction was significant ($p<0.05$) in WGF.

As a summary we reported here the variation in PA and PA ratios in white flour and wholegrain flour of a subset of 24 genotypes derived from crosses of Watkins landraces and Paragon.

## 3.5 DISCUSSION AND CONCLUSIONS

White flour and wholegrain flour wheat samples of a subset of 24 genotypes from lines derived from the Watkins diversity set, were processed and their PA concentrations were determined. A commercial kit was used for this purpose. Phytate ratios were calculated using data previously generated by ICP-MS.

The recovery of the assay was 60 and 43 % for harvest year 1 and 2 respectively, based on the reference material provided by the manufacturer.

PA concentration global mean (n=242) in 24 genotypes, two sites during two harvest years in white flour samples was 956 ± 677.8 mg $kg^{-1}$ (mean ± SD) with median of 825.1 mg $kg^{-1}$.

PA concentration in white flour samples was 1430.1 ± 553.8 mg $kg^{-1}$ in harvest year 1 and 414.7 ± 289.1 mg $kg^{-1}$ in harvest year 2. Harvest year 1 was notably higher than harvest year 2 and this could be because 54 out of 129 data values were lower than the calculated LOD and were replaced by half-LOD values.

Table 3-22 describes these samples, 7 out of 11 countries of origin were represented. Spain and Tunisia with the largest number of samples (12) followed by Australia, Canary Islands, Cyprus, Turkey and Morocco (8, 7, 6, 5 and 4 respectively). Burma, Portugal, Palestine and Greece did not have samples in this situation. Out of 54 samples, 31 were from RRes site and 23 from UoN site. The proportion from the total number of samples was RRes (31/114=0.27) and UoN (23/128=0.18). The imputed samples were distributed across 17 of the 24

genotypes studied and across the four levels of Fe-Zn established. The proportions of samples that were replaced by half-LOD were: HFe-HZn (23/72=0.32), LFe-HZn (10/71=0.14), HFe-LZn (6/27=0.22), LFe-LZn (15/72=0.21).

Table 3-22 Counts of samples of which the phytic acid concentration was lower than the LOD and were replaced by half-LOD value.

| Genotype | Country of origin | Site | n |
|---|---|---|---|
| PxW7 - 2 | Australia | RRes | 1 |
| PxW7 - 60 | Australia | RRes | 2 |
| PxW7 - 60 | Australia | UoN | 1 |
| PxW7 - 76 | Australia | RRes | 2 |
| PxW7 - 76 | Australia | UoN | 2 |
| PxW264 - 17 | Canary Islands | UoN | 1 |
| PxW264 - 50 | Canary Islands | RRes | 3 |
| PxW264 - 50 | Canary Islands | UoN | 3 |
| PxW291 - 23 | Cyprus | UoN | 1 |
| PxW291 - 75 | Cyprus | UoN | 3 |
| PxW291 - 75 | Cyprus | RRes | 2 |
| PxW216 - 88 | Morocco | RRes | 1 |
| PxW254 - 2 | Morocco | RRes | 3 |
| PxW273 - 21 | Spain | UoN | 1 |
| PxW273 - 71 | Spain | RRes | 3 |
| PxW273 - 71 | Spain | UoN | 1 |
| PxW546 - 20 | Spain | RRes | 1 |
| PxW546 - 24 | Spain | RRes | 1 |
| PxW685 - 36 | Spain | RRes | 3 |
| PxW685 - 36 | Spain | UoN | 2 |
| PxW811 - 30 | Tunisia | RRes | 3 |
| PxW811 - 30 | Tunisia | UoN | 3 |
| PxW811 - 83 | Tunisia | RRes | 3 |
| PxW811 - 83 | Tunisia | UoN | 3 |
| PxW299 - 87 | Turkey | RRes | 3 |
| PxW299 - 87 | Turkey | UoN | 2 |

LOD, Limit of Detection; n, number of samples; RRes, Rothamsted Research; UoN, University of Nottingham.

Wholegrain flour PA concentrations were quite similar in both harvest years, 9594 ± 1795 mg $kg^{-1}$ in harvest year 1 and 9122 ± 1345 mg $kg^{-1}$ in harvest year 2 (mean ± SD).

White flour PA concentrations were higher at UoN compared to RRes site 32.6 % higher in harvest year 1 and 54 % higher in harvest year 2. PA concentrations in wholegrain flour samples were 18.2 % higher at UoN site in harvest year 1 whereas in in year 2, PA concentrations were 6.3 % higher at RRes site. Suggesting the environmental factor was a strong influence for the concentrations of PA. In this study the soil management was slightly different in both sites in terms of the N fertilization rate and time of application and the crops in rotation, at Nottingham the previous crop in both years was oilseed rape and in Rothamsted the first year was wheat and in the second one was winter oats, details were described previously in the materials and methods section. Unfortunately in this study we were not aware if soil mineral analysis were performed.

Other authors have previously reported that environmental conditions are an important factor influencing PA concentrations (Branković, Dragičević, Dodig, Zorić, et al., 2015; M. Li et al., 2015; Raboy & Dickinson, 1993), therefore factors like the soil management could have affected the PA concentrations.

The lowest PA concentration in white flour samples in harvest year 1 across the two sites was the genotype PxW299 – 87. In harvest year 2 the lowest across the two sites, was genotype PxW291 – 75. Is important to mention that for harvest

year 2 the genotypes PxW811 – 83 (HFe-HZn from Tunisia), PxW264 – 50 (HFe-LZn from Canary Islands) and PxW811 – 30 (HFe-HZn from Tunisia) had actually lower values but these were the samples which values were replaced by half-LOD. The highest value in harvest year 1 was the genotype PxW546 – 25 (only data from UoN was available) and in harvest year 2, across the two sites, the highest was the genotype PxW546 – 20, Table 3-23 shows details of these genotypes.

Table 3-23 Summary of highest and lowest PA concentrations in white flour and wholegrain wheat of a panel of 24 genotypes derived from Watkins landraces and Paragon. Data are in mg $kg^{-1}$.

| | **White flour** | | | |
|---|---|---|---|---|
| | **Harvest year 1** | | **Harvest year 2** | |
| | **Lowest** | **Highest** | **Lowest** | **Highest** |
| **Genotype** | PxW299-87 | PxW546-25 | PxW291-75 | PxW546-20 |
| **PA (mg $kg^{-1}$)** | 876.4 | 2682.6 | 213.6 | 845.9 |
| **Country of origin** | Spain | Spain | Cyprus | Spain |
| | **Wholegrain flour** | | | |
| **Genotype** | PxW566-20 | PxW546-24 | PxW7-60 | PxW685-36 |
| **PA (mg $kg^{-1}$)** | 8112 | 10917.9 | 7145.4 | 10550.7 |
| **Country of origin** | Greece | Spain | Australia | Spain |

Cell colours represent the Fe-Zn level: High Fe-High Zn (red); Low Fe-High Zn (blue); High Fe-Low Zn (black); Low Fe-Low Zn (green).

Our results were in agreement to the reports of Norhaizan & Nor Faizadatul Ain (2009) in Malaysian wheat flour, 849.6 mg $kg^{-1}$ of PA. However, the authors did not explicitly say they worked with white flour. We also have similarities with the range stated by Tabajjoh (2011) in bread flours from factories in Iran 460-4380 mg $kg^{-1}$, although our mean for harvest year 2 is still way lower than these reports.

On the contrary, other investigations have presented values much higher than ours, for example, in Pakistani spring wheat straight-grade flour Anjum (2002) reported 2400 mg $kg^{-1}$ which is almost 2.5-fold our global mean for flour; in other studies, Kashlan (1990) reported PA of several extraction rates (ER %) of wheat flour used in Kuwait, the highest the percentage the more bran it contains, ER 95 %, 10630 mg $kg^{-1}$; ER 91 %, 7980 mg $kg^{-1}$; ER 86-89 %, 7380 mg $kg^{-1}$ and ER 78 %, 2380 mg $kg^{-1}$ (dry weight basis, DWB), even the lowest ER %, was higher than the reported in our study. Febles (2002) in refined flour reported a range of 2000-4000 mg $kg^{-1}$; Wu (2010) reported in Chinese hard winter wheat white flour a PA concentration of 1540-4000 mg $kg^{-1}$; and Tavajjoh (2011) in hand debranned bread flours in Iran, PA 4740-8650 mg $kg^{-1}$, whose work stressed the differences between machine processed flours and the hand processed ones, which tend to have more bran remains and consequently more phytic acid.

We compared our results to those reported in the PhyFoodComp 1.0 database (Dahdouh et al., 2018) for wheat white flour from different locations. India, 1230 mg $kg^{-1}$; New Zealand, 2552 mg $kg^{-1}$; Bangladesh, 2270 mg $kg^{-1}$; Turkey, 2143.2 mg $kg^{-1}$; Ireland, 1600 mg $kg^{-1}$; Bolivia, 2486.1 mg $kg^{-1}$; Kenya, 3200 mg $kg^{-1}$; Bangladesh, 1470 mg $kg^{-1}$, all reported values higher than in this study, Figure 3-16. One-way ANOVA showed significant differences among the countries of origin and the PA concentrations ($p<0.001$) in WF. For comparison sake, the mean value across all sites and years for the genotypes studied from Turkey was 563.4 mg $kg^{-1}$ which is 2.8-fold lower than the reported in the database. Turkey had in

fact the lowest mean PA in white flour across all sites and years, compared to Burma with 1839.9 mg $kg^{-1}$.

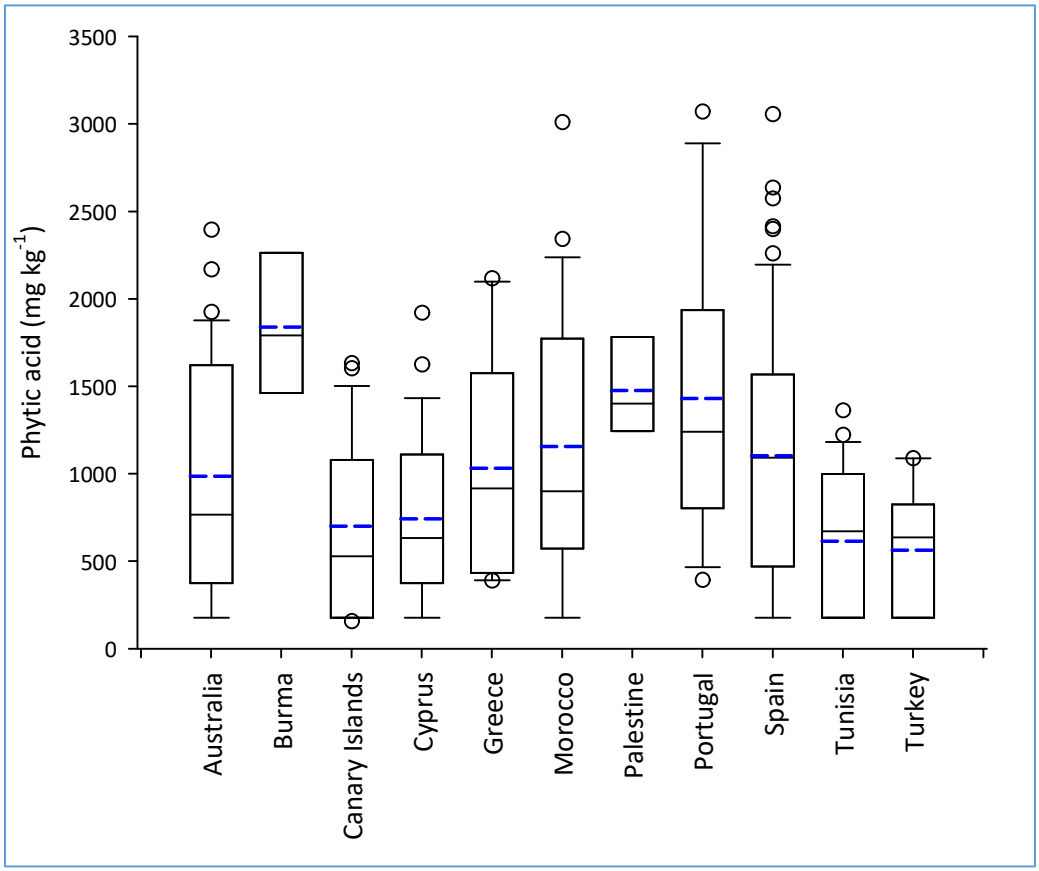



Figure 3-16 Phytic acid concentrations (mg $kg^{-1}$) in white flour by country of origin in a panel of 24 genotypes grown at two sites in the UK during two harvest years. Boxplot shows two mid-quartiles with the median drawn (solid black line) and mean (dotted blue line); whiskers are the 90$^{th}$ and 10$^{th}$ percentiles and circles are outliers. Australia, n=36; Burma, n=3; Canary Islands, n=24; Cyprus, n=27; Greece, n=12; Morocco, n=24; Palestine, n=3; Portugal, n=12; Spain, n=62; Tunisia, n=27; Turkey n=12.

The database reports concentration values as fresh weight, in our study we did not perform moisture content analysis but because our samples were weighed after being oven dried for at least 24 h before the determinations, they are

considered as dry weight. It should be noted that the concentrations just mentioned were all obtained by different analytical methods than the one we used in this study (Megazyme kit).

On the other hand, in our wholegrain flour samples the PA concentration global mean (n=242) in 24 genotypes, two sites during two harvest years was 9374 mg $kg^{-1}$. The lowest PA value was the genotype PxW566 – 20 in harvest year 1 and PxW7 – 60 in harvest year 2. The highest value harvest year 1 was in PxW546 – 24 whereas in harvest year 2 it was PxW685 – 36, Table 3-23 shows details of these genotypes.

Norhaizan & Nor Faizadatul Ain (2009) reported PA concentrations for processed food mee kuning (noodles), 28.6 mg $kg^{-1}$ and wheat kueh teow, 1100 mg $kg^{-1}$. It is not very clear if the noodles were cooked, food processing such as making noodles can contribute to reduce the PA concentrations and the heat treatment from cooking can even reduce the concentrations even more. The concentration reported for wheat was 8.5-fold lower than our global mean and although it was not specified, assuming it was wholegrain flour, it seems to be unusually low. However, this can be a result from the differences in the analytical methods. Anjum (2002) in Pakistani whole-wheat flour reported 23000 mg $kg^{-1}$ (or 2.23 %), which is 2.5-fold higher than our global mean and even 1.7-fold higher than the maximum value in our set (13220 mg $kg^{-1}$).

Wheat studies by Li (2011) reported in Chinese spring wheat a range of 730-1660 mg $kg^{-1}$ and in winter wheat between 600-1850 mg $kg^{-1}$ (DWB). Their maximum values were in average 35 % lower than the minimum in our set (4853 mg $kg^{-1}$).

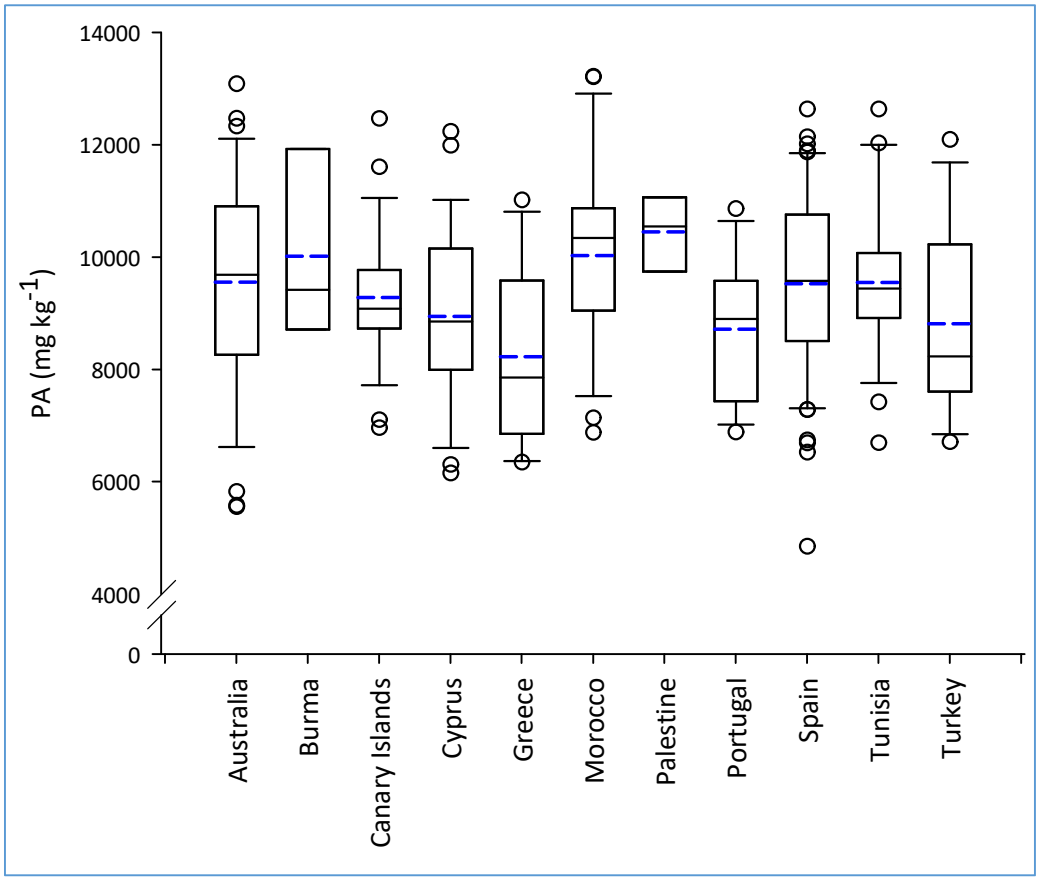



Figure 3-17 Phytic acid (PA) concentrations (mg $kg^{-1}$) in wholegrain flour by country of origin in a panel of 24 genotypes grown at two sites in the UK during two harvest years. Boxplot shows two mid-quartiles with the median drawn (solid black line) and mean (dotted blue line); whiskers are the 90$^{th}$ and 10$^{th}$ percentiles and circles are outliers. Australia, n=36; Burma, n=3; Canary Islands, n=24; Cyprus, n=27; Greece, n=12; Morocco, n=24; Palestine, n=3; Portugal, n=12; Spain, n=62; Tunisia, n=27; Turkey n=12.

More similar to our findings were those of Magallanes-López (2017) who reported in durum wheat varieties a range of 4620-9520 mg $kg^{-1}$. Febles (2000) reported 7740 mg $kg^{-1}$ in wheat grain from the Canary Islands, in our study the mean for the genotypes from the Canary Islands was 9285 ± 1208 mg $kg^{-1}$ (Figure 3-17); in a different study Febles (2002) reported whole flours (germ and bran added, as defined by the authors) 6000-10000 mg $kg^{-1}$; Wu (2010) in Chinese hard white winter wheat whole flour reported 9600-22200 mg $kg^{-1}$; (Tavajjoh et al., 2011) in bread flours in Iran reported in wholegrain a range of 7650-8590 mg $kg^{-1}$. One-way ANOVA showed significant differences among the countries of origin and the PA concentrations ($p=0.043$) in WGF.

Some accessions included in the PhyFoodComp 1.0 database (Dahdouh et al., 2018) for wholegrain wheat flour from different countries/regions are: Spain 9863.7 mg $kg^{-1}$ (very similar to our findings, 9529 ± 1632.3 mg $kg^{-1}$), Ethiopia 11029 mg $kg^{-1}$, Nigeria 11467 mg $kg^{-1}$, Europe (UK, France, Netherlands, Germany or Belgium) 5370 and 8140 mg $kg^{-1}$, USA 5557.2 mg $kg^{-1}$, Ireland 7700 mg $kg^{-1}$. The database expresses values as fresh weight, all our values are dry weight.

In WF the PA concentrations were significantly different across the genotypes ($p<0.001$), sites ($p<0.001$) and years ($p<0.001$). Similarly, in WGF the mean values for PA concentrations differed across the genotypes ($p<0.001$), sites ($p=0.001$) and years ($p=0.017$). Note that data from 5 genotypes (PxW233-80, PxW291-39, PxW398-18, PxW546-25 and PxW811-10, all from level 3 HFe-LZn) was missing from RRes in both harvest years and from UoN in harvest year 2.

The PA in this set of wholegrain samples was 48 % less than what we obtained in the hydroponic experiments, taking only the data from the standard treatments (p=0.25 mmol $L^{-1}$, Zn= 1 µmol $L^{-1}$).

Based on the ANOVA, the mayor source of variation for PA content in WF and WGF was the environment (55 and 15 %, respectively). Both WF and WGF similar genotype effects while the interaction accounted for more variability in WGF than in WGF.

It is not surprising that the environment has a large effect on the PA concentrations, providing is a complex trait. This might seem like bad news for breeders, however, it provides the chance to exploit genotypes with low PA content in specific environments or even manipulate/regulate the PA concentrations according to the environment. This coincides with what we observed in the hydroponic experiment, with wholegrain flour.

There were big differences between the years in WF, PA mean concentrations in harvest year 2 were 71 % lower compared to harvest year 1. In WGF the differences were not so drastic, harvest year had 5 % less PA compared to the first harvest year. Across both harvest years, the PA concentration was 32% lower in RRes compared to UoN in WF. For WGF, RRes had 6.3 % less PA compared to UoN.

We found significant differences in genotype and site regarding phytic acid-P/total P %. In white flour samples, genotype, site and year were all significant ($p<0.001$) but year had the greatest effect followed by the genotype. In wholegrain flour

samples, genotype ($p=0.030$) and year ($p<0.001$) were significant, and genotype had the greatest effect on the variation, whereas the site was not significant ($p=0.069$).

In WF the harvest year 1 had higher values (25.03 %, 31.43 %; RRes and UoN, respectively) compared to harvest year 2 (8.17 %, 11.54 %; RRes and UoN, respectively). Data from two year indicated that the percentages at UoN site were 38 % higher than those of RRes sites. In WGF, harvest year 1 had a similar tendency for RREs but not for UoN, (59.5 %, 65 %; RRes and UoN, respectively) compared to harvest year 2 (72.5 %, 63 %; RRes and UoN, respectively)

Tavajjoh (2011) reported in hand debranned bread flours from Iran, phytic acid-P/total P %=42.5-98.4 % and in mechanical processed flours 14.3-67.4 % whereas for wholegrain flour the authors found 51.5-97.2 %, greater than the global range reported in this study, 4.066-51.55 % in flour (if compared to the mechanical processed samples) but similar for wholegrain flour samples 35.22-102.6 %. From the data reported by Tang (2008) we calculated the phytic acid-P/total P in break flour, 64.09 % which is higher than our flour samples. Lolas (1976) in 38 cultivars of wheat in the USA, reported a range of 61.7-79.9 %. Guttieri (2006c) reported in wholegrain 72 % and 64 % in two different sites. They also demonstrated significant differences in the P uptake regarding the irrigation and the different P concentrations between different years.

In our study the major source of variance in phytic acid-P/total P % in WF was notably the environment followed by the genotype and only a little was accounted by the interaction. In WGF the greatest variance driver was also the environment but not as much as in WF, followed by the interaction and the genotype. The model described more of the variance in WF than it did on WGF indicating there might be other variables involved.

This could indicate that the climatic conditions and soil management and composition play a big part in the phosphorus uptake and could play an important role in the distribution and composition of the P storage, the effect of the environmental effect had already been demonstrated previously for PA (Branković, Dragičević, Dodig, Zorić, et al., 2015).

PA and P had a strong positive relationship in white flour (r=0.878), followed by a moderate relationship with Mg, r=0.680 and Fe, r=0.62; $p<0.001$. The linear regression calculated to predict PA from P in white flour was significant and explained 74.4 % of the variability. Additionally, P had significant positive relationship with Mg, r=0.742; Fe, r=0.642; K, r=0.407; Zn, r=0.404 ($p<0.001$); Ca, r=0.208 ($p<0.01$); a negative relationship with Cu, r= -0.239 and not significant with Mn, r= -0.023 (p=0.723).

PA and P had a positive and significant relationship in wholegrain flour samples although not as strong as in white flour, and actually rather weak (r=0.495), PA and Mg, Zn and Fe were also significant but weak relationships. The linear

regression calculated to predict PA from P in wholegrain flour was significant but explained only 24.1 % of the variability. Contrary to what we observed in white flour, P had significant associations but the strength of the relationship was different, for example with K, r=0.504 and Mg, r=0.357 ($p<0.001$); with Zn, r=0.182 ($p=0.004$); Ca, r=0.160 ($p=0.013$), and negative relationships with Cu, r= -0.267 ($p<0.001$) and Mn, r= -0.208 ($p=0.001$), whereas no significant relationship was detected with Fe, r= -0.080 ($p=0.215$).

Lolas (1976) had previously reported a positive relationship between PA and total P in wheat grains (0.9682), which is higher than what we report here. This also supports the fact that it is possible to estimate the PA from a total P determination.

Tavajjoh (2011) observed a relationship between PA and Mg, r=0.59 and contrasting to our findings, with Mn, r=0.61 the authors even reported that up to 55 % of the PA concentration could be explained by Mn concentration and protein percentage; moreover Zn or Cu on a simple linear equation could predict 61 % of PAxCa:Zn and using Cu in a cubic model increased the prediction power of PAxCa:Zn up to 78 %, the authors also described an equation that could predict 92 % of the phytic acid-P/total P %.

Stangoulis (2007) in rice previously reported significant positive correlations between phytate and inorganic phosphorus (r=0.746), total P (r=0.996), Fe (r=0.528), Zn (r=0.402), Cu (r=0.330) and Mn (r=0.559). Magallanes-López (2017)

in durum wheat reported significant correlation between PA and Zn in two different environments.

Similar to our results, Tang (2008) reported significant positive relationships of P with Mg, Cu, Fe and K but not with Zn, Mn or Ca in break flours of Chinese wheat cultivars. In wholegrain, the authors reported relationship of P with Mg, Zn, Cu, Mn and Fe, but not with K and Ca. Morgounov (2007) in spring and winter wheat genotypes from Central Asia also reported a positive relationship of P with Mn and Zn.

The strong relationships between PA and these minerals can be explained by the mechanisms of PA storage in plants. Globoids are the structures where phytic acid is found, and they are localized mainly in the protein storage vacuoles in the aleurone layer in wheat. This also supports why the PA is more abundant in wholegrain flour than in white flour. The size of the globoids depends on the PA content in the grain. L. Bohn (2007) demonstrated the connection between several minerals such as K, Mg, Ca, and Fe. Therefore, it is widely known that PA main role is the storage of P and minerals, it has been suggested that it could act as a sink and reservoir of minerals. It is believed that this mechanism may have been useful in wild plants, before domestication and it might not be critical in cultivated crops (Raboy et al., 2001). Furthermore, recently published evidence identified genomic regions where genetic markers for Zn, Fe, Cu, Mn and P concentrations in grain are co-located (Cu et al., 2020).

Differences in the strength of the relationship between PA and P in both fractions might be related to differences in the histochemical distribution of the minerals and PA. In wheat grain, phytic acid accumulates predominantly in the aleurone layer whereas phytase, Fe, and Zn accumulate mostly in the outer layer (I. Cakmak et al., 2010), but a considerable portion is also found in the starchy endosperm (Tang et al., 2008).

PA molar ratios were calculated for Ca, Fe, and Zn as well as PAxCa:Zn. Our results indicated significant differences between the years, sites and genotypes ($p<0.001$) in white flour and wholegrain flour samples.

To evaluate the degree in which the phytic acid concentrations would affect the bioavailability of Ca, Fe and Zn we took as reference the critical values proposed for PA:Ca >0.24 (Morris & Ellis, 1985), PA:Fe >1 (Hallberg et al., 1989), PA:Zn >15 (Morris & Ellis, 1989; Sandberg et al., 1987; Turnlund et al., 1984) and PAxCa:Zn >200 (Bindra et al., 1986; Davies et al., 1985).

Based on evidence, the WHO categorized diets according to the potential availability of their zinc, among other considerations, high zinc availability will be given in diets that are low in phytic acid content and the PA:Zn ratio is <5; moderate between 5-15 and low if the ratio is >15. These bioavailability levels correspond to 50 %, 30 % and 15 % of zinc absorption (WHO & FAO, 2006; World Health Organization, 1996).

**White flour:** UoN site ratios were higher than those in RRes site. We observed 65 % increase for PA:Ca, 32 % for PA:Fe, 87.7 % for PA:Zn and 74.5 % for PAxCa:Zn. Mean values of PA:Ca ratios were above the critical value at UoN but not at RRes site. PA:Fe ratios were above the critical values in all sites. PA:Zn and PAxCa:Zn ratios were lower than the critical values in both sites, therefore good bioavailability for Zn can be expected. All the ratios calculated were significantly higher on the first year compared to the second year. PA:Ca 4-fold, PA:Fe 2.6-fold, PA:Zn 2.98-fold and PAxCa:Zn 3.3-fold.

In this study the range phytate ratios in 24 genotypes, in two sites during two years in white flour samples were, PA:Ca=0.0320-0.882 (n=235), PA:Fe=1.005-14.54 (n=234), PA:Zn=1.167-26.27 (n=235) and PAxCa:Zn=5.569-182.3, Table 3-16.

Our results were similar to those of Norhaizan & Nor Faizadatul Ain (2009), in Malaysian wheat flour with the exception of PA:Ca= 3.07 which was higher than our findings. The PhyFoodComp 1.0 database (Dahdouh et al., 2018) present values for white flours from different countries, Table 3-24, all were between the ranges obtained in this study with the exception of Turkey, which had reported higher values of PA:Fe which is opposite to our findings where the genotype PxW299-87 from Turkey had the lowest PA:Fe (3.8), Table 3-16.

The results of Tavajjoh (2011) in hand debranned flours were higher than those observed in this study whereas ratios of the flour processed in a mill were closer to our results. This is consistent with the fact that most of the PA is found in the

aleurone layer, therefore if some of the bran was left while debranning by hand, the PA detected would be higher, Table 3-24. Our samples were processed in a mill and we can see that the results are comparable.

Table 3-24 White flour phytic acid ratios obtained by previous studies.

| Region/Country | PA:Fe | PA:Zn | PAxCa:Zn | Reference |
|---|---|---|---|---|
| Malaysia | 8.06 | 21.5 | | (Norhaizan & Nor Faizadatul Ain, 2009) |
| India | 5.88 | 13.85 | | (Dahdouh et al., 2018) |
| New Zealand | | 21.6 | | |
| Bangladesh | | 14.51 | | |
| Bangladesh | 4.61 | 9.39 | | |
| Turkey | 22.56 | | | |
| Ireland | 11.57 | 23.92 | | |
| Bolivia | 4.39 | 17.73 | | |
| Iran (hand debranned flours) | | 16.2-48.9 | 80-270 | (Tavajjoh et al., 2011) |
| Iran (mill processed flours) | | 3.0-24.6 | 20-180 | |
| Bangladesh (refined wheat flour) | | 12.45 | | (Chowdhury et al., 2002) |

Significant differences for phytate ratios were observed between genotypes. Genotype PxW546-25 had the highest ratios in all minerals, Table 3-16. This genotype had also the highest PA mean value in harvest year 1 whereas there was

no data for this genotype in harvest year 2. This genotype was reported to have the highest grain protein content, 18.2 % (Khokhar et al., 2020). Although it has not been largely studied, there could be a relationship between the protein content and phytic acid content or could affect indirectly the protein content, some pleiotropic effects seen in low phytic acid mutants are low grain weight, lower field emergence. In rapeseed cultivars, the protein extraction from seeds is impaired by the presence of tightly bound phytins and tannins (Wanasundara et al., 2016) and in wheat the aleurone has around ~15 % of the wheat total protein, which is where the PA primarily allocates. Raboy (1991) reported protein was highly and positively correlated in winter wheat.

In this study we identified 10 out of 24 genotypes had a PA:Ca ratio <0.24, PxW264 – 50, PxW299 – 87, PxW811 – 83, PxW291 – 75, PxW811 – 30, PxW685 – 36, PxW273 – 71, PxW264 – 17, PxW273 – 21, PxW291 – 23, which means they would have good Ca bioavailability. These 10 genotypes would comply with having good bioavailability of Ca and Zn but it is likely that the Fe bioavailability would be affected.

**Wholegrain flour:** Our results indicated significant differences between the sites for all calculated ratios ($p<0.001$), between years only PA:Fe ($p<0.001$) and between genotypes ($p<0.001$) only PA:Ca and PAxCa:Zn. The ranges obtained for each ratio were: PA:Ca=0.575-2.859 (n=240), PA:Fe=12.51-43.47 (n=240), PA:Zn=11.12-56.58 (n=240) and PAxCa:Zn=142.3-786.6 (n=240).

The wholegrain Fe and Zn concentrations of Paragon were 29.6 and 34.4 mg $kg^{-1}$, averaged across both sites in 2015– 16, respectively (Khokhar et al., 2020). Comparing to our results in the hydroponics P experiment (P treatment 3, 0.25 mmol $L^{-1}$, n=16) Paragon had a Fe mean concentration of 50.2 mg $kg^{-1}$ and a Zn mean concentration of 30.8 mg $kg^{-1.}$ In our hydroponic Zn experiment (Zn treatment 2, 1 µmol $L^{-1}$, n=17) mean Fe concentration was of 34.0 mg $kg^{-1}$ and Zn concentration of 17.8 mg $kg^{-1}$, all averaged across the three years. Although Fe and Zn were somewhat higher in the hydroponics experiment, the ratios were much lower in this subset of genotypes, and this could be explained by the high availability of phosphorus in hydroponics.

Comparing to our previous hydroponic experiment with Paragon, the ratios were lower in this subset of 24 genotypes: compared to the P experiment, PA:Ca ratios were 16.6 % lower; PA:Fe, 6.6 lower and PA:Zn, 37.7 % lower. Compared to the Zn experiment, PA:Ca ratios were 28.2 % lower; PA:Fe, 29.8 lower and PA:Zn, 63.5 % lower.

Values reported in the PhyFoodComp 1.0 database for whole wheat flour in several countries are in Table 3-25. In comparison to our values, only the PA:Zn reported in Ethiopia exceeds our values. The results by Magallanes-López (2017) are within the range of our samples although our maximum values were much larger than theirs and the reports by Tavajjoh (2011) were lower than ours.

All the ratios were higher at UoN site compared to RRes site. We observed a 22 % increase for PA:Ca, 16 % for PA:Fe, 59 % for PA:Zn and 39 % for PAxCa:Zn. All molar ratios calculated were above the critical values for a good bioavailability, indicating that the mineral absorptions of all wholegrain flour samples from the genotypes analysed are compromised by the levels of phytic acid. PA:Fe ratios were 39 % higher on the first year compared to the second year.

Table 3-25 Wholegrain phytic acid ratios obtained by previous studies.

| Region/Country | PA:Fe | PA:Zn | PAxCa:Zn | Reference |
|---|---|---|---|---|
| **Ethiopia** | 19.8 | 73.2 | | PhyFoodComp 1.0 database (Dahdouh et al., 2018) |
| **USA** | | 25.1 | | |
| **Ireland** | 27.9 | 50.2 | | |
| **Mexico (durum wheat)** | 12.1-29.6 | 16.9-23.6 | | (Magallanes-López et al., 2017) |
| **Iran** | | 23.4-45.6 | 130-420 | (Tavajjoh et al., 2011) |
| **Bangladesh** | | 27.25 | | (Chowdhury et al., 2002) |

Significant differences for PA:Ca and PAxCa:Zn were observed among genotypes, but not for PA:Fe and PA:Zn). In summary, all genotypes had ratios that exceed the recommended critical values for good Ca, Fe and Zn bioavailability.

Some authors have reported highly significant effect of the environment, genotype and their interaction on PA ratios, for example, (Magallanes-López et al.,

2017) reported environment influence was high for PA:Fe ratios, and PA whereas PA:Zn was more dependent on the genotype * environment interaction. In our study for PA:Fe and PA:Zn the variability was more determined by the environment than by the genotype and the interaction. These results could help to find or develop varieties adapted to different environmental conditions and that could also be a good source for increasing Fe and Zn.

# 4. PHYTIC ACID DETERMINATION IN INDIAN WHEAT VARIETIES GROWN UNDER HOSTILE CONDITIONS

## 4.1 INTRODUCTION

In India and in large parts of the world, the cultivation land is affected by salinity and/or sodicity/alkalinity. New wheat varieties are developed in India through a systematic testing procedure. Variety trials, grain multiplication and the field evaluation trials involves evaluation for yield and contributing traits, disease incidence, agronomic practices and grain quality traits. However, most of these trials used for screening of new varieties, are performed in normal soils while trials in marginal soils are not routinely conducted. Examining traits in these marginal soils may help to identify and select varieties with good performance and with salt-tolerance characteristics.

Zinc (Zn) deficiency is a common micronutrient deficiency in wheat growing in many climatic regions, particularly in calcareous soils of arid and semiarid regions. It causes severe decreases in grain yield and quality. High levels of $CaCO_3$ and pH and low levels of soil moisture and organic matter are major reasons for widespread occurrence of Zn deficiency and other minerals including Fe. These soil factors are known to limit mobility and availability of soil-Zn or fertilizer-Zn to plant roots (Ismail Cakmak & Marschner, 1986; Lantican et al., 2003; H. Marschner, 1993)

The interactions between phosphorus content in soils and zinc availability to plants are highly complex. Generally, with increase in soil content or supply of fertilizer phosphorus, plant uptake of zinc decreases more or less sharply and often beyond a level which can be attributed to dilution effects due to growth enhancement. In contrast, extractable zinc in soils is either not or only slightly decreased by high phosphorus supply (H. Marschner, 1993).

Crops growing on soils low in readily available P are often found to exploit more P from the soil, it has been observed that plant roots induce changes in the physico-chemical equilibrium in the rhizosphere to adapt to conditions of P deficiency. Manske (2001) found that under conditions of P deficiency, wheat plants utilized the adsorbed P more efficiently for grain yield formation and that phosphorus uptake by the above ground biomass was highest in calcareous soils with P fertilization. Erdal (2002) found that increases in P and PA concentrations in seeds under Zn deficiency could be ascribed to Zn deficiency-enhanced uptake and shoot accumulation of P. Other researcher have found that Zn deficiency, can lead to P poisoning. In monocotyledonous Gramineae plants, an inadequate Zn supply could lead to increased P concentration in plant tissues, and eventually P poisoning (Webb & Loneragan, 1988).

In this study, we analysed six genotypes derived from a wide genetic background grown at three different environments in India. We selected genotypes with low and high concentrations of P and Zn. Additionally, we calculated the phytic acid molar ratios on these genotypes to see how the PA concentrations could

potentially affect the bioavailability of minerals such as Fe and Zn in wheat products. We hypothesized that giving the Zn deficient nature of hostile soils, the genotypes grown there would have higher concentrations of P and therefore of phytic acid.

## 4.2 AIMS AND OBJECTIVES

To determine phytic acid (PA) concentrations in a subset of Indian-adapted wheat genotypes grown on a wide range of soil types without addition of Zn fertiliser and establish the antinutritional effect on Fe and Zn bioavailability. In this study we have the following objectives: 1) describe the variability in wholegrain PA in a subset of Indian genotypes; 2) estimate the bioavailability of Fe and Zn in wholegrain flours; 3) examine the relationship between P, Zn and PA concentrations in wholegrain flours; 4) examine the effect of genotype, environment and their interaction on PA concentrations and PA ratios.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Background information of germplasm, experimental sites and field management

Previous to this study, a panel of 36 elite wheat genotypes comprising *Triticum aestivum* L. (n = 34) and Triticum durum (n = 2) was selected for study under field conditions. These genotypes represent a diverse genetic background with adaptations to a range of climatic and soil environments (Khokhar et al., 2017).

The study was carried out at six field sites in major wheat growing areas of India, during rabi (winter) seasons in 2013/14 and 2014/15. Two sites were selected from within an agro-climatic region of the North Western Plains Zone (NWPZ); four sites were selected from the North Eastern Plains Zone (NEPZ), two sites from each of two different agro-climatic regions in NEPZ. Within each region, one site was selected to represent a 'normal' soil, the other a 'hostile' soil. The NWPZ sites were, (1) IIWBR, Karnal, Haryana (HR); (2) IIWBR, Hisar (HR); NEPZ were, (3) Narendra Deva University of Agriculture and Technology (NDUA&T), Kumarganj, Faizabad, Uttar Pradesh (UP), reclaimed site; (4) NDUA&T, Kumarganj, Faizabad (UP), sodic site; (5) Uttar Banga Krishi Viswavidyalaya (UBKV), Pundibari, Cooch Behar, West Bengal (WB); (6) UBKV, Regional Research Sub-Station (RRSS), Mathurapur, Malda (WB). Sites 2, 4 and 5 represent 'hostile' soils (pH range 4.5–9.5), and sites 1, 3 and 6 represent 'normal' soils (pH range 7.2–8.3).

Wheat was sown in rabi season in November/December and harvested during April/ May in 2013/14 and 2014/15, at all the sites. After conventional operations, including field preparation, fertilizing, disking, levelling and furrowing, seeds were

manually placed in four rows per plot at 25 cm spacing between rows. The plot length was 2.5 m. The number of seeds sown for each genotype was the same for each plot (~624 seed). The plots were arranged in a simple lattice design (6x6) with two replicates according to standard IIWBR practices. Fertilizers were applied at a rate of 150 kg N, 60 kg $P_2O_5$ and 40 kg $K_2O$ $ha^{-1}$ as urea, triple super phosphate (TSP) and muriate of potash (MOP), respectively. All fertilizers, including one third of the N, were applied uniformly in the field during final land preparation. One third of the N was top dressed at nodal root initiation (NRI) stage, ~21 days after sowing (DAS), and the remaining N was applied at the time of second irrigation, ~45 DAS. Experiments were irrigated when required to avoid water stress to the plants, typically on three occasions, to bring the soil moisture close to the field capacity during NRI, booting and grain filling stages. Weeds were controlled manually at 30 DAS by hand weeding and then crops were protected from pests and disease as required. Crops were harvested at maturity, sun-dried and then threshed.

### 4.3.2 Grain material sampling and preparation

From the field experiments detailed in the previous section, a subsample of six genotypes of wheat (Triticum aestivum L.) were selected based on their phosphorus and zinc concentration and the availability of the seed in the laboratory. Two out of the three agro-climatic zones were considered, NWPZ (North-Western Plains Zone) and NEPZ (North-Eastern Plains Zone) Table 4-1.

Based on the pH of the soil, the sites were catalogued in two status: normal and hostile.

We selected one normal site (Karnal, pH 7.5) and two hostile sites (Hisar, pH 8.1; and Kumarganj-sodic, pH 9.5), Table 4-1. Only the seed from the 2014 harvest was used. Two field replications from Karnal were available and only one from Hisar and Kumarganj-sodic sites.

Table 4-1 Soil conditions in which the selected genotypes were grown.

| | Zone | Soil type | Status | Soil pH |
|---|---|---|---|---|
| **Hisar** | NWPZ | Saline | Hostile | 8.1 |
| **Karnal** | NWPZ | Normal | Not Hostile | 7.5 |
| **Kumarganj-sodic** | NEPZ | Sodic | Hostile | 9.5 |

NWPZ, North-Western Plains Zone; NEPZ, North-Eastern Plains Zone

Grain digestion and multielemental analysis was previously conducted and it is described in Khokhar et al., (2017). Results of the determinations for relevant minerals in this investigation are reported in the next section.

For the phytic acid analysis three technical replicates were used from each genotype and its corresponding site. Only for Karnal site we used six technical replicates. In total, 72 samples were analysed using the phytic acid kit (phytic acid/total phosphorus; Megazyme International Ireland, Bray Co. Wicklow, Ireland) and the concentration is reported here as mg $kg^{-1}$.

The genotypes used here were categorised based on their P and Zn concentrations. We analysed three genotypes with the highest P and Zn concentration and three with the lowest P and Zn concentration (respect to the original 36 genotype panel). The genotypes selected were: BH_1146, KHARCHIA_65 and KRL_3-4 (High P-High Zinc) and HD_2932, HW_2044, WH_1021 (Low P-Low Zn).

### 4.3.3 Summary statistics of the sample set

Data for phosphorus, zinc and grain yield were obtained by ICP-MS and analysed previously by Jaswant Khokhar. The analysis and construction of figures presented on this investigation were done using mean values (data processed), Table 4-2. For more details consult Table 9-19.

Table 4-2 Mineral concentrations of six genotypes grown at three sites in India during 2013/14 and 2014/15. Means of four replicates per genotype at Karnal and Hisar sites and three replicates per genotype at Kumarganj-sodic site. From Khokhar et al., (2017).

| Genotype | Site | Ca | Fe | P | Zn |
|---|---|---|---|---|---|
| BH_1146 | HISAR | 428.3 | 48.7 | 3558.8 | 36.0 |
| BH_1146 | KARNAL | 407.3 | 47.4 | 3850.2 | 35.8 |
| BH_1146 | KUMARGANJ-SODIC | 298.2 | 42.3 | 3812.7 | 29.5 |
| HD_2932 | HISAR | 437.8 | 37.6 | 2923.9 | 25.6 |
| HD_2932 | KARNAL | 369.9 | 30.9 | 2829.3 | 23.9 |
| HD_2932 | KUMARGANJ-SODIC | 338.1 | 39.8 | 3433.7 | 24.4 |
| HW_2044 | HISAR | 447.4 | 39.2 | 2900.0 | 24.5 |
| HW_2044 | KARNAL | 486.2 | 38.9 | 3470.5 | 29.2 |
| HW_2044 | KUMARGANJ-SODIC | 310.1 | 38.9 | 3353.7 | 23.5 |
| KHARCHIA_65 | HISAR | 408.2 | 45.0 | 3800.4 | 40.2 |
| KHARCHIA_65 | KARNAL | 413.2 | 49.3 | 3877.0 | 38.2 |
| KHARCHIA_65 | KUMARGANJ-SODIC | 338.0 | 45.7 | 3869.5 | 34.9 |
| KRL_3-4 | HISAR | 451.1 | 45.3 | 3034.5 | 27.2 |
| KRL_3-4 | KARNAL | 587.3 | 55.9 | 4056.3 | 40.9 |
| KRL_3-4 | KUMARGANJ-SODIC | 342.9 | 40.2 | 3789.8 | 28.3 |
| WH_1021 | HISAR | 338.6 | 35.0 | 2988.9 | 29.0 |
| WH_1021 | KARNAL | 397.6 | 43.3 | 3318.5 | 28.7 |
| WH_1021 | KUMARGANJ-SODIC | 361.2 | 46.5 | 3586.1 | 26.2 |

Table 4-3 Grain yield (t $ha^{-1}$) of six genotypes and three sites in India. Data are means from two years (n=4); from Khokhar (2017)*.

| Genotype | Hisar | Karnal | Kumarganj-sodic | Across site |
|---|---|---|---|---|
| **BH_1146** | 2.99 | 4.33 | 1.55 | 2.96 |
| **HD_2932** | 3.55 | 5.33 | 1.65 | 3.51 |
| **HW_2044** | 3.03 | 5.71 | 1.33 | 3.36 |
| **KHARCHIA_65** | 1.64 | 1.75 | 1.10 | 1.50 |
| **KRL_3-4** | 2.90 | 1.51 | 1.07 | 1.83 |
| **WH_1021** | 3.99 | 6.19 | 1.17 | 3.78 |
| **Across genotype** | 3.02 | 4.14 | 1.31 | 2.82 |

*Supplementary Table 2 Raw data of yield and component traits of 36 genotypes evaluated at six sites over two years (2013/14 and 2014/15).

### 4.3.4 Phytic acid determination

The wholegrain flour phytic acid concentration of 72 samples were determined using a commercial kit (Phytic acid/total phosphorus; Megazyme International Ireland, Bray Co. Wicklow, Ireland), with slight modifications. The assay measures phytic acid as phosphorus released by a phytase and an alkaline phosphatase. The principle of the method can be found in Figure 2-10 on chapter 2.

The procedure was done as described previously in page 78: Phytic acid/total phosphorus quantification in Paragon wheat leaf and grain grown in NFT hydroponic system.

### 4.3.5 Phytate molar ratio determination

Phytic acid to mineral ratios were obtained calculating the millimoles of PA dividing the concentration in mg $kg^{-1}$ by its molecular weight (MW) in g $mol^{-1}$ and equally with each of the mineral elements. Next, the resulting millimoles of PA are divided by the millimoles of the corresponding mineral. Molecular weight used were PA=660.04, Ca=40.08, Co=58.93, Cu=63.55, Fe=55.85, K=39.1, Mg=24.31, Mn=54.94, P=30.97 and Zn=65.4 g $mol^{-1}$.

### 4.3.1 Statistical analysis

Data management and analysis were performed using GenStat 19th Edition (VSN International Ltd, Hemel Hempstead, UK), Microsoft Excel 2016 and Sigma Plot Version 13.0 (Systat Software, Inc.).

### 4.3.2 Data cleaning and tiding

Raw data processing was done as described in the following paragraphs. Data corresponds to the 1st (Run 0) and 2nd (Run 1-3) set of Indian wheat grain.

The first set was processed as proof of concept on May 2018 and the second set was analysed on February 2019. Only the wholegrain flour was analysed.

The average of each of the phosphorus standards was calculated, Table 4-4. Then the absorbance values of the blanks were introduced in the calculation software provided by the manufacturer assuming 1 g of sample for calculation purposes.

Table 4-4 Phosphorus calibration curve for phytic acid determination.

| Phosphorus Standard | Phosphorus (µg) | Abs 1 | Abs 2 | Abs 3 | Mean | SD |
|---|---|---|---|---|---|---|
| STD0 | 0 | 0.03 | 0.03 | 0.04 | 0.035 | 0.0007 |
| STD1 | 0.5 | 0.12 | 0.12 | 0.12 | 0.216 | 0.0008 |
| STD2 | 2.5 | 0.46 | 0.47 | 0.47 | 0.465 | 0.0040 |
| STD3 | 5 | 0.90 | 0.91 | 0.88 | 0.896 | 0.0120 |
| STD4 | 7.5 | 1.33 | 1.34 | 1.33 | 1.334 | 0.0062 |

Abs, Absorbance; SD, standard deviation.

The LOD was obtained using the standard deviation of the differences of absorbance (delta-Abs Phosphorus at 650nm) of four blank solutions, blank for Run0 was included with the rest (run 1-3) because in this run only one blank was analysed, see Table 9-20.

The LOD was calculated as three times the standard deviation (0.0039 units of absorbance) and it was equivalent to 21.7 mg $kg^{-1}$ of total phosphorus or 76.96 mg $kg^{-1}$ of phytic acid.

None of the values in this analysis were under the calculated LOD. Therefore, no values had to be substituted.

The recovery of the assay was calculated according to the values obtained by the oat flour reference material provided by the manufacturer in the phytic acid assay kit. The measured values (n=4) are shown in Table 9-21.

According to the manufacturer the established concentration of oat flour is 17700 mg $kg^{-1}$. The mean of the measured values was 9486.52 ± 852.65 mg $kg^{-1}$ of phytic acid. The recovery percentage was 53.60 % and the relative standard deviation (RSD) was 8.99 %.

Paragon laboratory reference material (LRM) was also processed but only in the second set. The raw data is presented in Table 9-22. The mean value was 7507.87 ± 198.24 mg $kg^{-1}$ of phytic acid, mean ± SD. The RSD was 2.64 %.

To eliminate any outliers, the global mean of phytic acid plus five times the standard deviation was calculated. No outliers were identified for these samples. The calculated LOQ was 18264.31 mg $kg^{-1}$ of phytic acid. Summary statistics are presented in Table 9-23.

## 4.4 RESULTS

### 4.4.1 Phytic acid concentrations results

The mean value of phytic acid concentration across all genotypes and sites (n=72) was 8371.86 ± 1978 mg $kg^{-1}$ (mean ± SD) with a range of 4306.79 to 11962.87mg $kg^{-1}$ and a median value of 8515 mg $kg^{-1}$.

The mean values and standard deviations for phytic acid concentration are presented in Figure 4-1.

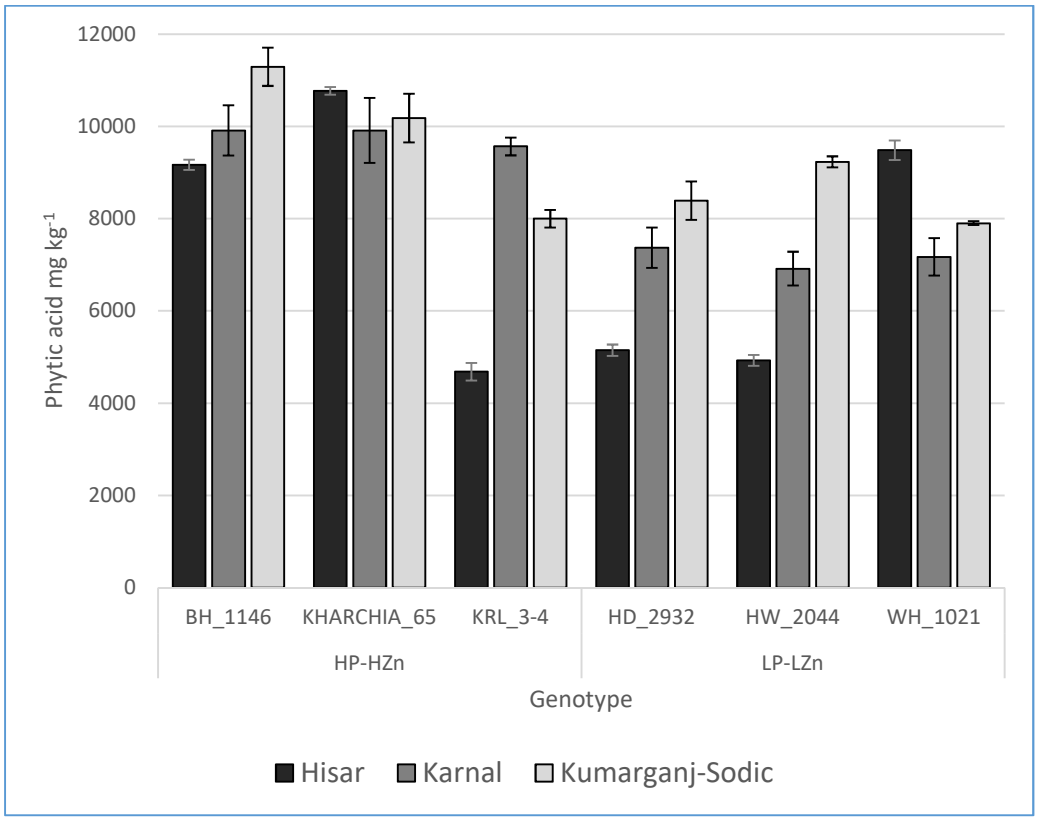



Figure 4-1 Phytic acid concentration (mg $kg^{-1}$) in 6 wheat genotypes grown at three different sites in India in 2013/14. Data are means ± SE bars. Karnal (n = 6), Hisar and Kumarganj-sodic (n = 3). HP-HZn, High Phosphorus-High Zinc; LP-LZn, Low Phosphorus-Low Zinc.

The PA concentrations by genotype across all three sites were: BH_1146 10073 ± 1244 mg $kg^{-1}$; KHARCHIA_65 10196 ± 1280 mg $kg^{-1}$; KRL_3-4 7955 ± 2117 mg $kg^{-1}$; HD_2932 7073 ± 1469 mg $kg^{-1}$ HW_2044 7000 ± 1708 mg $kg^{-1}$ and WH_1021 7935 ± 1200 mg $kg^{-1}$. Six replications per genotype at Karnal site and three replications per genotype at Hisar and Kumarganj-sodic (Kum-S), means ± SD.

The mean phytic acid values by site were: Hisar, 7365 ± 2577 mg $kg^{-1}$; Karnal, 8478 ± 1720 mg $kg^{-1}$ and Kumarganj-sodic, 9168 ± 1359 mg $kg^{-1}$.

Averaged across sites, the genotype KHARCHIA_65 had the highest concentration of phytic acid (10195.8 ± 1279.89 mg $kg^{-1}$) meanwhile the lowest concentration was for the genotype HW_2044 (6999.62 ± 1708.4 mg $kg^{-1}$).

Two-way analysis of variance (ANOVA) was conducted to test for differences in phytic acid concentration due to genotype and site factors. Differences between sites and genotypes were considered statistically significant at $p<0.05$. If significant, the mean values were compared using the Fisher's Least Significant Difference (LSD). The Shapiro-Wilk test for normality indicated a normal distribution for these data (Test statistic W: 0.9900; p = 0.845).

Genotype, site as well as their interaction were statistically significant ($p<0.001$) for the phytic acid concentration in wholegrain flour. Genotype (LSD 1 %= 987.7), site (LSD 1 %=734.4) and interaction genotype x site (LSD 1 %=1794).

Genotype accounted for the largest proportion of the variance (44 %) followed by the interaction (29 %) and lastly the environment (11 %), Table 4-5.

Table 4-5 Effects of genotype, environment and their interaction (G x E) expressed as % of the total sum of squares from ANOVA analysis for phytic acid in wholegrain flour of six genotypes grown at three different sites in India in 2013/14.

| Source of variation | df | SS | SS (%) | MS | p |
|---|---|---|---|---|---|
| **Genotype** | 5 | 121892346.2 | 43.9 | 24378469.2 | <0.001 |
| **Environment** | 2 | 30065145.09 | 10.8 | 15032572.5 | <0.001 |
| **G x E** | 10 | 81624959.64 | 29.4 | 8162496.0 | <0.001 |
| **Residual** | 54 | 44341122.3 | 16.0 | 821131.9 | |
| **Total** | 71 | 277923573.2 | | 3914416.5 | |
| SE = 906.2 | CV = 10.8 % | Adj. $R^2$ = 79 % | | | |

df, degrees of freedom; SS, sum of squares; SS (%), SS as percentage to the total; MS, mean squares; p, probability; SE, standard error; Adj. $R^2$, adjusted $R^2$ (%); CV, coefficient of variation (%); Environment = Site.

Phosphorus-Zinc status was significantly associated with the levels of PA ($p<0.001$, LSD 1 %= 1056.6). HighP_HighZn genotypes had significantly higher concentrations of PA (9408 ± 1870 mg $kg^{-1}$, n=36) compared to the LowP_LowZn genotypes (7336 ± 1495 mg $kg^{-1}$, n=36).

Mean PA values averaged across the three sites for genotypes KHARCHIA_65 and BH_1146 were significantly higher compared to the rest of the genotypes, Figure 4-2.

Among the sites, Hisar had significantly ($p=0.019$) lower levels of PA (7364.56 mg $kg^{-1}$) compared to Karnal (8477.62 mg $kg^{-1}$). Between Karnal and Kumarganj-sodic sites (9167.64 mg $kg^{-1}$) there were no significant differences.

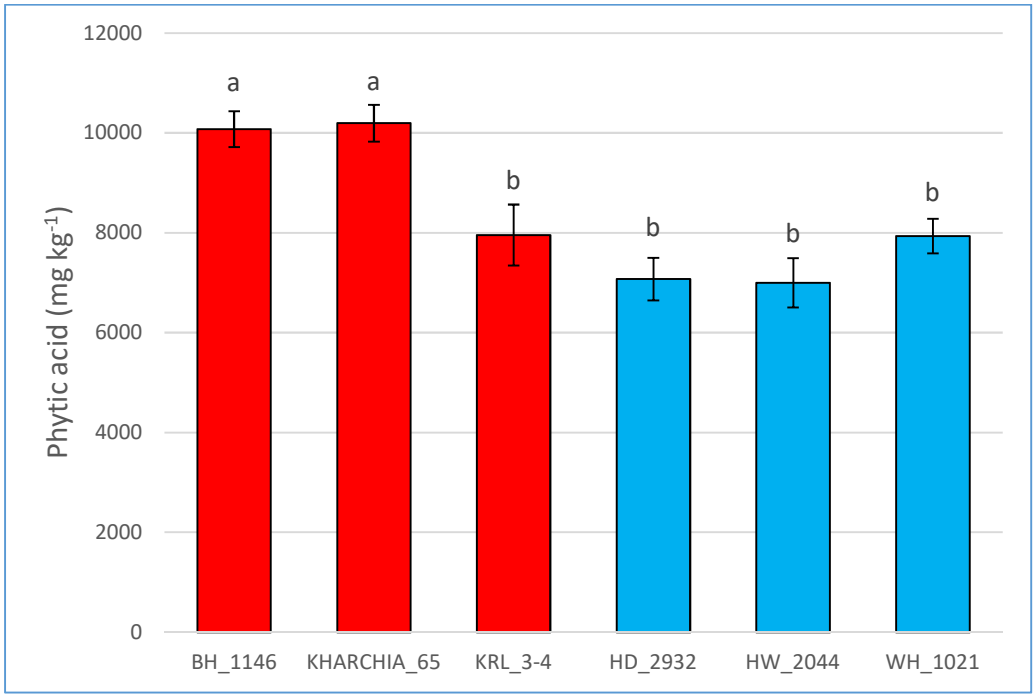



Figure 4-2 Phytic acid means of six genotypes grown at three different sites in India in 2013/14. Means followed by a common letter are not significantly different by Fisher's LDS test at the p=0.01 level of significance. Means ± SE bars. Red coloured bars are HighP-HighZn genotypes; blue coloured bars are LowP-LowZn genotypes. n=12.

#### *4.4.1.1 Dilution effect? Data correction by grain yield*

The grain yield (GYD) for this data set was significantly different between the sites and genotypes ($p<0.001$), see Table 4-3 for values. Following a similar approach as McDonald (2008), we ran a correlation analysis to identify if PA and other mineral concentrations were influenced by the grain yield, Table 4-6.

The correlations were not very strong ($r<0.8$) but were all inversely related. Therefore, to consider a possible dilution effect the PA values (mg $kg^{-1}$) were multiplied by the grain yield (t $ha^{-1}$) and divided the result by 1000 to obtain PA expressed in kg $ha^{-1}$. Results of this data correction are in Figure 4-3.

Table 4-6 Simple correlation coefficients between grain phytic acid and other mineral concentrations and grain yield.

| PA/mineral concentration | Grain yield |
|---|---|
| Phytic acid | -0.411 ** |
| Phytic acid-P/total P % | -0.186 |
| Ca | 0.107 |
| Fe | -0.465 ** |
| P | -0.521 ** |
| Zn | -0.317 * |

*p<0.01; **p<0.001

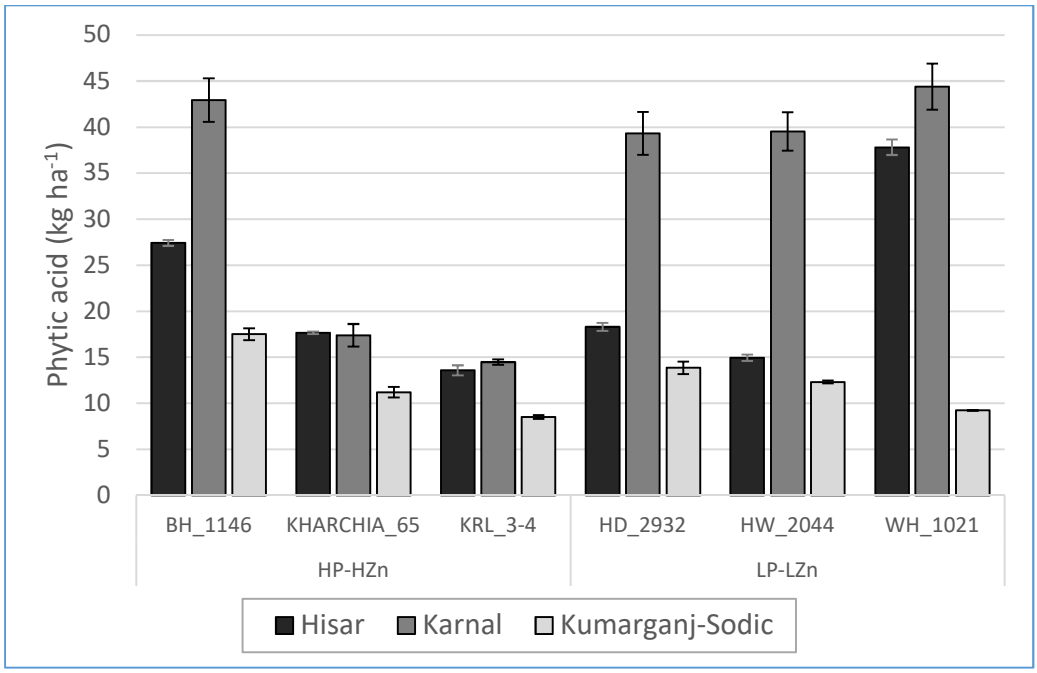



Figure 4-3 Phytic acid means corrected by grain yield of six genotypes grown at three different sites in India in 2013/14. Data are means ± SE bars. Karnal (n = 6), Hisar and Kumarganj-sodic (n = 3). High Phosphorus-High Zinc, HP-HZn; Low Phosphorus-Low Zinc, LP-LZn. Data are in kg $ha^{-1}$.

However, the residuals of the corrected data were not normal (Test statistic W: 0.948, p=0.005). Therefore to be able to analyse it by ANOVA we log-transformed the data. This normalised the data and then we ran a two-way ANOVA. The results indicated significant genotype, environment (site) and their interaction. Compared to the data without the correction by grain yield, the source of the variation changed, the environment accounted now for most of the variance (48 %) followed by the genotype (34 %) and lastly the interaction G x E (15 %), Table 4-7. To identify the differences among the genotypes the Fisher LSD test was applied, results are shown in Figure 4-4.

Table 4-7 Effects of genotype, environment and their interaction (G x E) expressed as % of the total sum of squares from ANOVA analysis for phytic acid (PA) in wholegrain flour of six genotypes grown at three different sites in India in 2013/14. Data was normalised by multiplying PA concentrations by the grain yield and log transformed.

| **Source of variation** | **df** | **SS** | **SS (%)** | **MS** | **p** |
|---|---|---|---|---|---|
| Genotype | 5 | 1.43904 | 34.6 | 0.28781 | <0.001 |
| Environment | 2 | 1.99560 | 47.9 | 0.99780 | <0.001 |
| G x E | 10 | 0.61441 | 14.8 | 0.06144 | <0.001 |
| Residual | 54 | 0.11482 | 2.8 | 0.00213 | |
| Total | 71 | 4.16387 | | 0.05865 | |
| SE = 0.04611 | CV = 3.5 % | Adj. $R^2$ = 96.4 % | | | |

df, degrees of freedom; SS, sum of squares; SS (%), SS as percentage to the total; MS, mean squares; p, probability; SE, standard error; Adj. R2, adjusted R2 (%); CV, coefficient of variation (%); Environment = Site.

To interpret and plot the data, PA values were back transformed using antilog. Across the three sites, genotype KRL_3-4 had the lowest PA concentrations (12.76 kg $ha^{-1}$) followed by KARCHIA_65 (15.9 kg $ha^{-1}$). The highest concentration corresponded to genotype WH_1021 (33.95 kg $ha^{-1}$). The sites differed significantly from each other. Karnal site had the highest mean PA concentrations (33 kg $ha^{-1}$) followed by Hisar (21.6 kg $ha^{-1}$) and Kumarganj-sodic site (12.1 kg $ha^{-1}$).

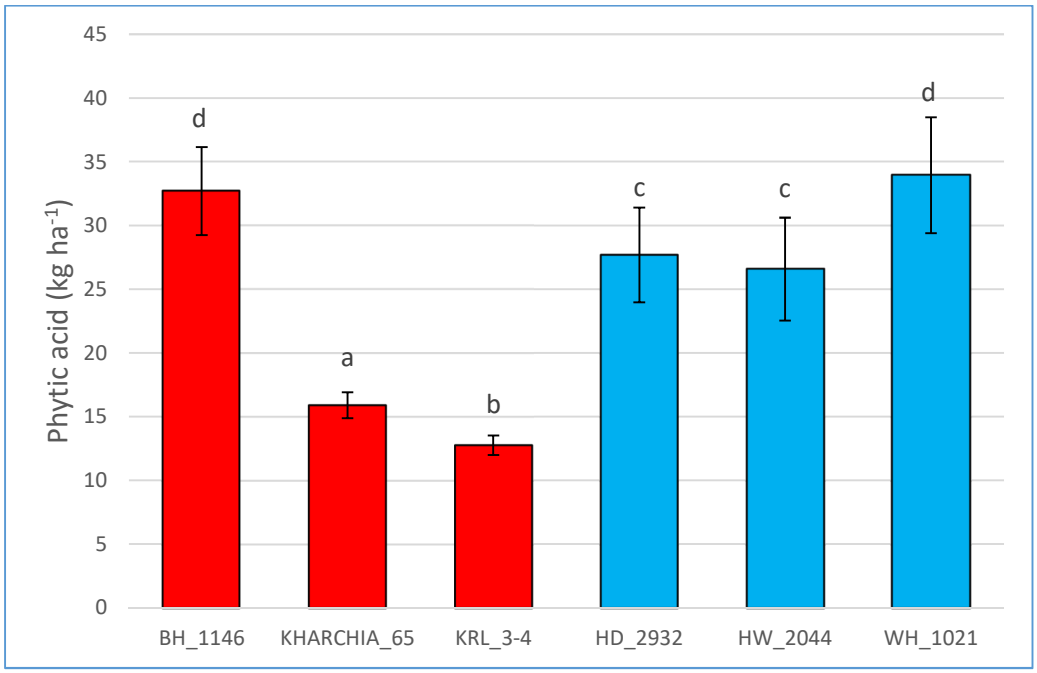



Figure 4-4 Phytic acid concentration (kg $ha^{-1}$) in 6 wheat genotypes grown at three different sites in India in 2013/14. Means followed by a common letter are not significantly different by Fisher's unprotected LDS test at the p=0.01 level of significance. Means ± SE bars. Red coloured bars are HighP-HighZn genotypes; blue coloured bars are LowP-LowZn genotypes. n=12.

We observed that the group of genotypes with the status LowP-LowZn had higher mean PA concentrations (29.40 ± 14.2 kg $ha^{-1}$) compared to the HighP-HighZn genotypes (20.45 ± 11.4 kg $ha^{-1}$).

### 4.4.2 Phytic acid-P as percentage of the total P (Phytic acid-P/total P %)

Phytic acid-P/total P refers to the proportion of P that is found as phytic acid and expressed as a percentage. In our wholegrain samples from Indian wheat this was in average 67.2 %, varied between 40.02 to 92.48 % with a median of 66.7 %.

Two-way ANOVA showed significant genotype ($p<0.001$, LSD 1 %=7.857), site ($p=0.0160$, LSD 5 %= 4.387) and genotype x site interaction ($p<0.001$, LSD 1 %=14.27). The genotype and interaction accounted for most part of the variation, 25 % and 46 % respectively, whereas the site effect accounted for 4 %. Figure 4-5 shows means for each genotype and site and the pooled mean of that particular genotype in all sites.

Averaged across all the sites, KRL_3-4 was the genotype with the lowest percentage (59.02 %) and the highest was BH_1146 (75.36 %). All genotypes averaged, showed Hisar, 63.9 % and Karnal, 70 %; were not significantly different as well as Karnal and Kumarganj-sodic, 71%; while Hisar and Kumarganj-sodic were significantly different.

Differences between the High-Low P-Zn status were not significant: HP-HZn, 69.7 %; LP-LZn, 64.8 %. There were also not significant differences between hostile and normal soils, but we did however observed that the hostile soils despite having significantly ($p<0.001$) less concentrations of PA compared to the normal soils, they tended to have a higher proportion of the total P as phytic acid (70.4 %) compared to the normal soil (64.5 %).

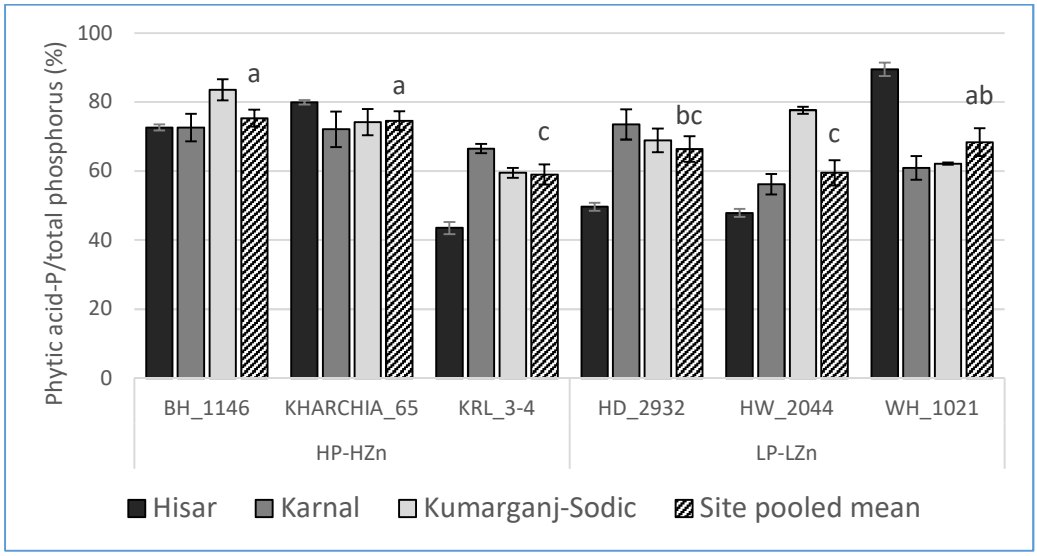



Figure 4-5 Phytic acid-P/total P % of six genotypes grown at three different sites in India in 2013/14. Means ± SE bars. High Phosphorus-High Zinc, HP-HZn; Low Phosphorus-Low Zinc, LP-LZn. Patterned bar represents the average of the three sites for the corresponding genotype. Means followed by a common letter are not significantly different by Fisher's LDS test at the p=0.01 level of significance.

### 4.4.3 Phytic acid and mineral interactions

A correlation analysis was performed to investigate if there were any relationships between the phytic acid concentrations and those of Ca, Fe, P and Zn. For these analysis the PA data used was the non-corrected by GYD.

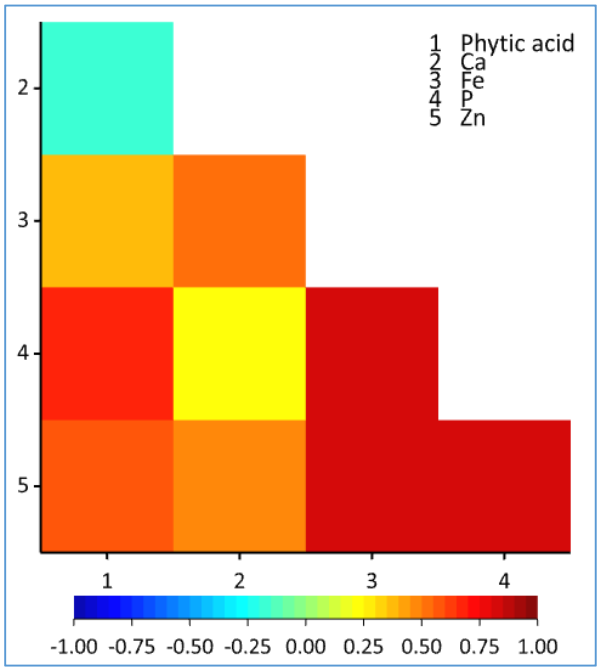



Figure 4-6 Relationships between phytic acid and other mineral elements. Data from six genotypes grown at three sites in India during two years. Phytic acid data from 2013/14 only. Data are in mg $kg^{-1.}$

The analysis of pooled data showed that phytic acid had a positive significant relationship with P ($r=0.677$, $p<0.001$), Zn ($r=0.595$, $p<0.001$) and Fe ($r=0.399$, $p<0.001$), but not with Ca ($p=0.127$). Calcium did have a positive relationship with Fe ($r=0.531$, $p<0.001$) and Zn ($r=0.499$, $p<0.001$) and similarly, Zn and Fe also correlated ($r=0.825$, $p<0.001$), this has been discussed previously (Khokhar et al., 2018). Besides phytic acid, phosphorus also had a strong and positive relationship with Fe ($r=0.810$, $p<0.001$), Zn ($r=0.809$, $p<0.001$), and a weaker relationship with Ca ($r=0.240$, $p=0.042$), Figure 4-6.

A simple linear regression was carried out to see if the P concentrations explained the PA concentrations. The regression was significant ($p<0.001$) and described by

the equation y = 3.705x – 4517.809 which explained the 51.5 % of the variance observed, Figure 4-7. It remains to be investigated if this relationship is maintained when correcting the data with the grain yield. In this study it was not possible to do so because the raw data was not available.

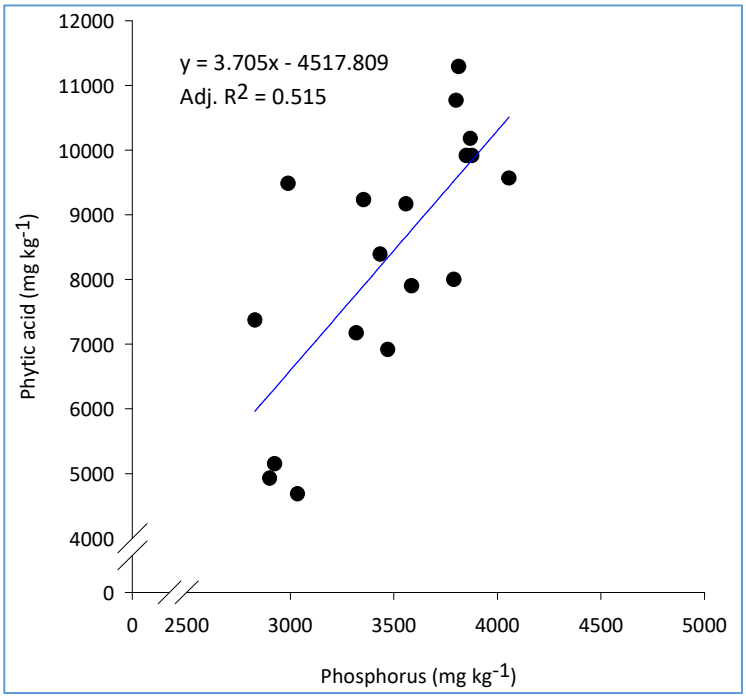



Figure 4-7 Simple linear regression explaining the relationship between phosphorus and phytic acid (PA) concentrations in wholegrain flour wheat. PA data points are means of three replicates per genotype per site and P data are means of four replicates per genotype at Hisar and Karnal and three at Kumarganj-sodic.

In the regression plot we can observe that there are three clusters, this is a consequence of using the mean values of P instead of the raw data. The three clusters correspond to the three sites. Mean P concentrations per site were: 3201, 3567 and 3641 mg $kg^{-1}$, for Hisar, Karnal and Kumarganj-sodic sites respectively.

Same correlation analysis were carried out using the site specific data to explore if these relationships were maintained on each site separately, Figure 4-8.

There were strong associations between PA and Zn. P, Zn and Fe were also strongly associated in all the sites. Only at Karnal P there was a medium-strong association with Ca, whereas Fe had associations with Ca at the three sites. Zinc was correlated with Ca only at Karnal and also only at this site was Fe correlated with Fe. P and PA were associated at Hisar and Karnal but not at the sodic site. Lastly, PA and Ca had a negative association only at the hostile soils (Hisar/Kumarganj-sodic), see Table 4-8. Note that these correlations were done using the data without correction for grain yield.

Using a Kruskal-Wallis one-way ANOVA and Mann Whitney U test with the corrected values (kg $ha^{-1}$), a comparison of the mineral concentrations among the sites was done. Compared to the normal site (Karnal), both Hisar and Kumarganj-sodic (hostile sites) had significantly ($p=0.001$) lower concentrations of Ca, Fe, P, Zn and PA in wholegrain. Equally, comparing hostile vs normal soils, the hostile soils had significantly ($p=0.001$) lower concentrations of Ca, Fe, P, Zn and PA in wholegrain.

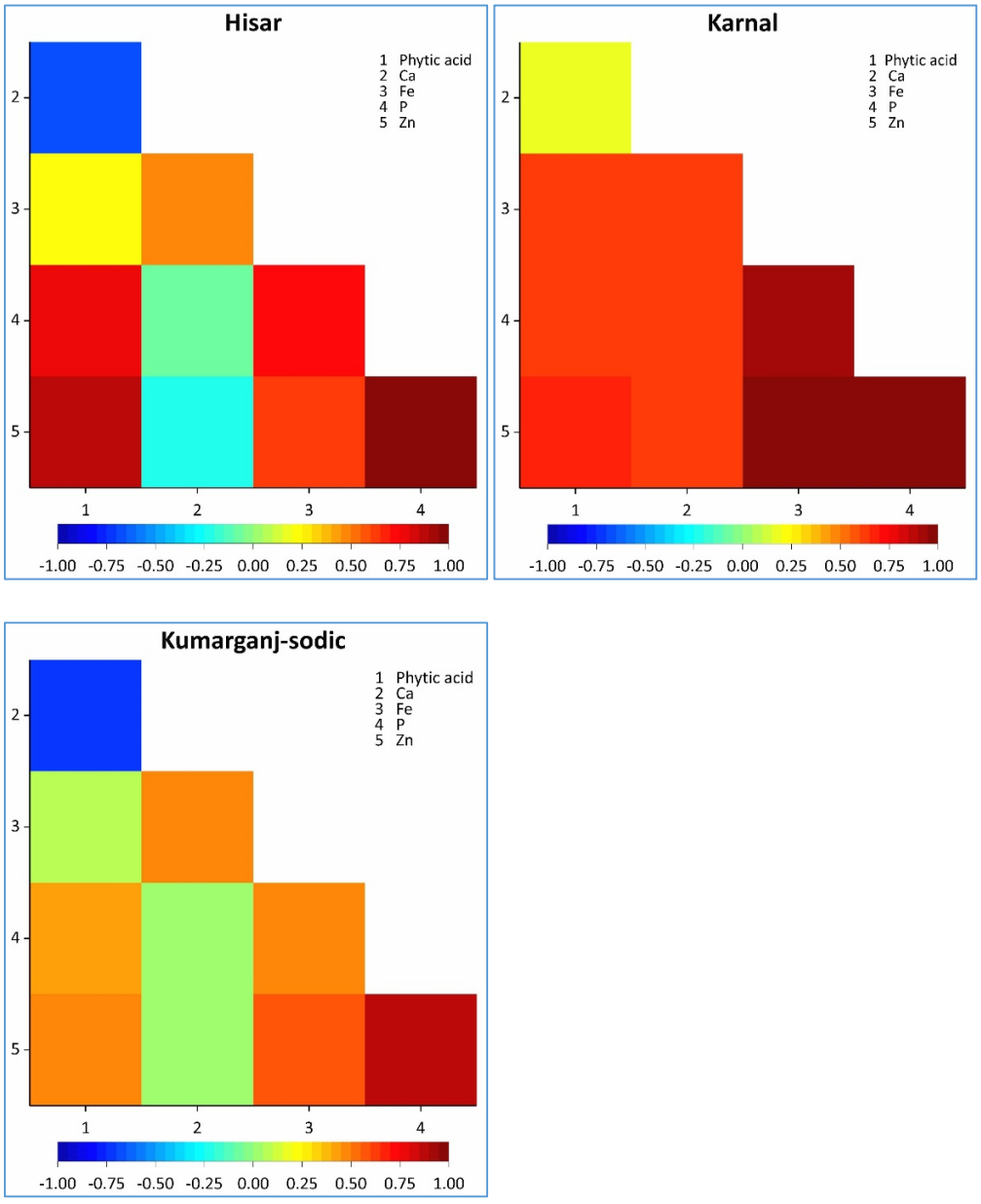



Figure 4-8 Relationships between phytic acid and other mineral elements. Data from six genotypes grown at three sites in India during two years. Phytic acid data from 2013/14 only. Data are in mg $kg^{-1.}$ Colour corresponds to the strength and direction of the correlation from strongly negative (dark blue) to strongly positive (dark red).

Table 4-8 Correlation coefficients among PA and Ca, Fe, P and Zn of 6 Indian wheat genotypes grown at three different sites in India. PA is data is from 2013-14 only. Ca, Fe, P and Zn concentrations are data from 2013-14 and 2014-15. From non-corrected data (mg $kg^{-1}$).

| | PA | Ca | Fe | P |
|---|---|---|---|---|
| | | **Hisar** | | |
| **Ca** | -0.672** | | | |
| **Fe** | 0.21 | 0.477* | | |
| **P** | 0.770*** | -0.065 | 0.712*** | |
| **Zn** | 0.861*** | -0.23 | 0.623** | 0.985*** |
| | | **Karnal** | | |
| **Ca** | 0.182 | | | |
| **Fe** | 0.605*** | 0.629*** | | |
| **P** | 0.634*** | 0.616*** | 0.950*** | |
| **Zn** | 0.688*** | 0.603*** | 0.961*** | 0.974*** |
| | | **Kumarganj-sodic** | | |
| **Ca** | -0.729*** | | | |
| **Fe** | 0.095 | 0.487* | | |
| **P** | 0.406 | 0.025 | 0.498* | |
| **Zn** | 0.490* | 0.033 | 0.590** | 0.896*** |

*p<0.05; **p<0.01; ***p<0.001

### 4.4.4 Phytic acid molar ratios

The phytic acid mineral molar ratios were calculated dividing the moles of PA (MW=660.04 g $mol^{-1}$) by the moles of Ca, Fe and Zn accordingly. A good bioavailability of the minerals observed if the ratios are below the critical values: PA:Ca, <0.24; PA:Fe, <1; PA:Zn, <15 and PA x Ca:Zn, <200.

There were no significant differences between the high and low P-Zn status of the genotypes, except for PA:Ca. We found that the PA:Ca ratios were lower for the HighP-HighZn genotypes (p=0.015), Table 4-9.

Table 4-9 General ANOVA showing differences between the high and low P-Zn status of 6 genotypes grown at three different sites in India, n=36.

| | PA:Ca | | PA:Fe | | PA:Zn | | PA x Ca:Zn | |
|---|---|---|---|---|---|---|---|---|
| | HP-HZn | LP-LZn | HP-HZn | LP-LZn | HP-HZn | LP-LZn | HP-HZn | LP-LZn |
| **Mean ratio** | 1.411 | 1.172 | 16.8 | 16.3 | 26.4 | 27.7 | 272.1 | 266.4 |
| **p** | 0.015 | | 0.614 | | 0.318 | | 0.566 | |

HP-HZn, High Phosphorus-High Zinc; LP-LZn, Low Phosphorus-Low Zinc.

Only PA:Ca and PA x Ca:Zn had significant differences between the hostile and normal soils, Table 4-10. Normal site performed better for PA:Ca ratios but the hostile soils did better for the PA x Ca:Zn ratios.

Table 4-10 General ANOVA showing differences between the PA ratios of 6 genotypes and the soil status in which they were grown in India, n=36.

| | PA:Ca | | PA:Fe | | PA:Zn | | PA x Ca:Zn | |
|---|---|---|---|---|---|---|---|---|
| | Hostile | Normal | Hostile | Normal | Hostile | Normal | Hostile | Normal |
| **Mean ratio** | 1.401 | 1.183 | 16.73 | 16.42 | 28.23 | 25.86 | 255.7 | 282.8 |
| **p** | 0.026 | | 0.735 | | 0.083 | | 0.005 | |

Hostile soils, Hisar and Kumarganj-sodic; Normal site, Karnal.

KRL_3-4 in Hisar was the genotype that had the lowest phytic acid ratios for all Ca, Fe and Zn, although only PA x Ca:Zn ratio was lower than the critical value of 200, meaning for this genotype at this site there would be a good bioavailability of zinc, Table 4-11.

Table 4-11 Highest and lowest phytic acid ratios.

| Ratio | Lowest | Site | Mean ± SD | Highest | Site | Mean ± SD |
|---|---|---|---|---|---|---|
| **PA:Ca** | KRL_3-4 | Hisar | 0.63 ± 0.044 | BH_1146 | Kum-S | 2.3 ± 0.146 |
| **PA:Fe** | KRL_3-4 | Hisar | 8.7 ± 0.61 | WH_1021 | Hisar | 22.9 ± 23.1 |
| **PA:Zn** | KRL_3-4 | Hisar | 17.1 ± 1.19 | HW_2044 | Kum-S | 39 ± 0.87 |
| **PA x Ca:Zn** | KRL_3-4 | Hisar | 192.4 ± 13.43 | KRL_3-4 | Karnal | 340 ± 335.2 |

Red font indicates HighP-HighZn genotype and blue font indicate LowP-LowZn genotype. Kum-S, Kumarganj-sodic.

Two-way ANOVA analysis for PA:Ca ratios showed the genotype (LSD 1 %=0.1498), site (LSD 1 %=0.1114) and the genotype x site interaction (LSD 1 %=0.2722) were significant ($p<0.001$). The overall mean was 1.33 with a range of 0.63 ± 0.04 to 2.30 ± 0.15. Figure 4-9 shows the mean ratios and significant differences within the genotypes and within the sites.

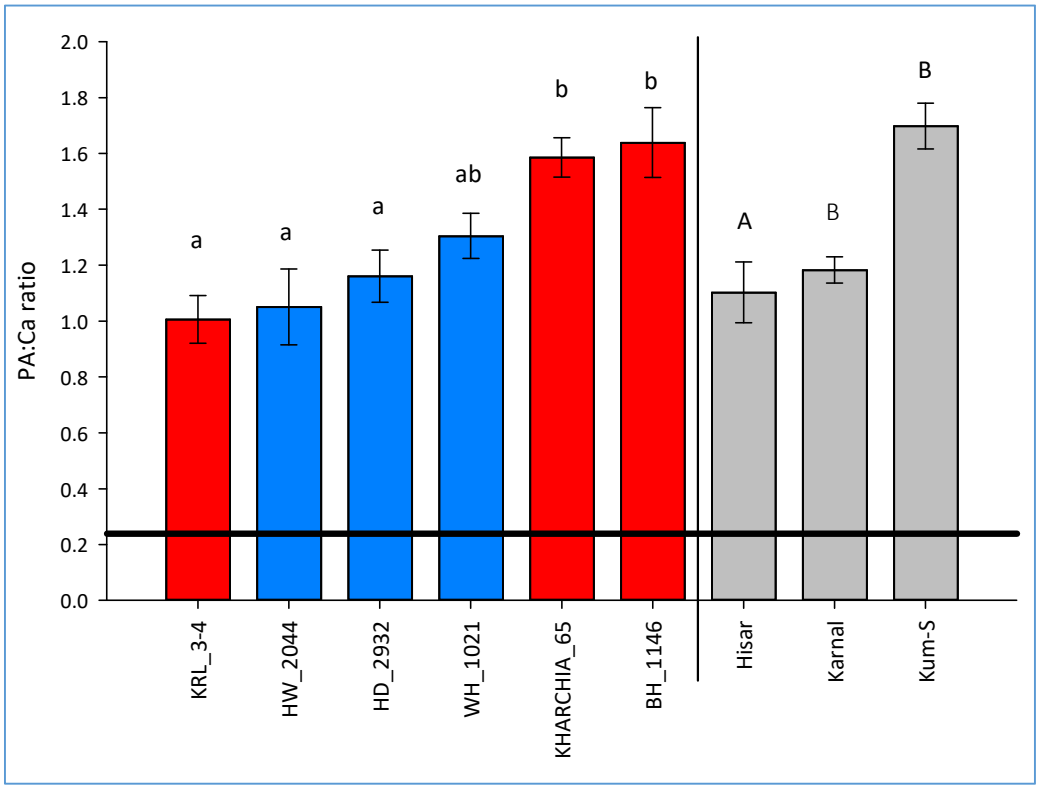



Figure 4-9 One-way ANOVA of PA:Ca ratios. Means ± SE bars. Left side of the vertical line are ratio means by genotype averaged across the three sites. Red bars, HighP-HighZn genotype; blue bars, LowP-LowZn genotype. Means followed by a common lowercase letter are not significantly different by Fisher's LDS test at the p=0.01 level of significance. Genotypes are sorted from lowest to highest mean. Right side of the vertical line are the ratio means by site averaged across the six genotypes. Means followed by a common uppercase letter are not significantly different by Fisher's LDS test at the p=0.01 level of significance. Sites are sorted from lowest to highest mean. Horizontal line at y=0.24 represents the critical value for PA:Ca ratio. Kum-S, Kumarganj sodic site.

For PA:Fe ratios the genotype (LSD 1 %=1.962), site (LSD 1 %=1.459) and the genotype x site interaction (LSD 1 %=3.565) were significant (p<0.001). The overall mean was 16.62 with a range of 8.74 ± 0.61 to 22.9 ± 0.88. Figure 4-10 shows the mean ratios and significant differences within the genotypes and within the sites.

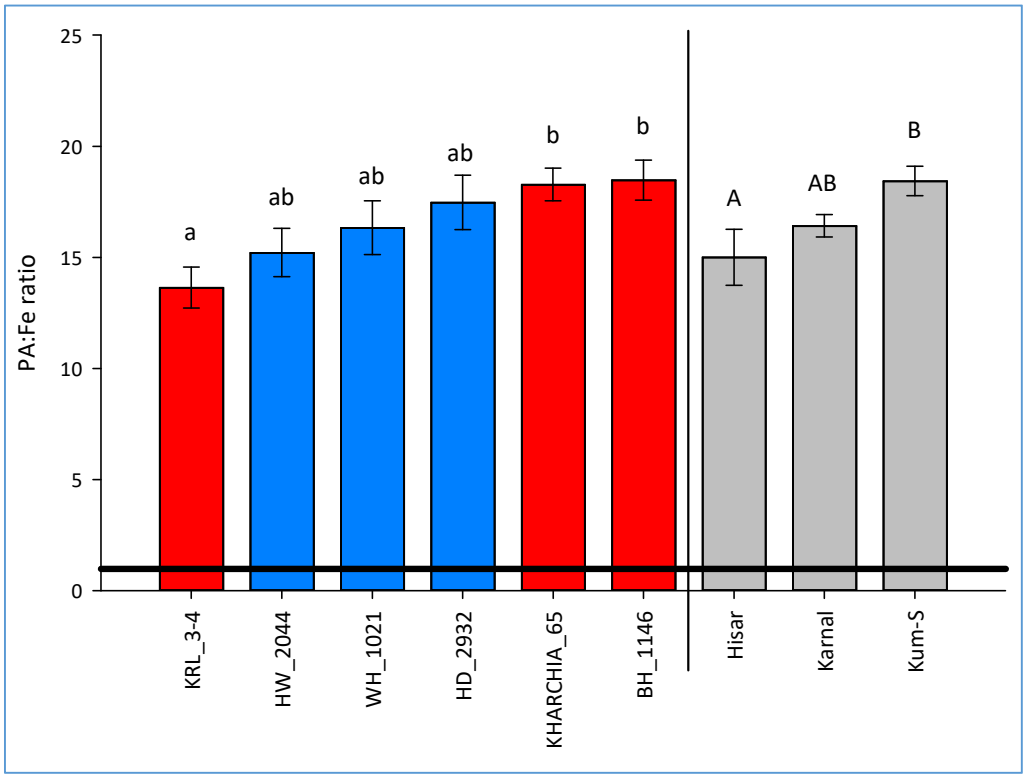



Figure 4-10 One-way ANOVA of PA:Fe ratios. Means ± SE bars. Left side of the vertical line are ratio means by genotype averaged across the three sites. Red bars, HighP-HighZn genotype; blue bars, LowP-LowZn genotype. Means followed by a common lowercase letter are not significantly different by Fisher's protected LDS test at the p=0.01 level of significance. Genotypes are sorted from lowest to highest mean. Right side of the vertical line are the ratio means by site averaged across the six genotypes. Means followed by a common uppercase letter are not significantly different by Fisher's protected LDS test at the p=0.05 level of significance. Sites are sorted from lowest to highest mean. Horizontal line at y=1 represents the critical value for PA:Fe ratio. Kum-S, Kumarganj sodic site.

PA:Zn ratios showed significant genotype (LSD 1 %=3.091), site (LSD 1 %=2.298) and genotype x site interaction (LSD 1 %=5.614) were significant (p<0.001). The overall mean was 27.44 with a range of 17.09 ± 1.19 to 38.95 ± 0.87. Figure 4-11 shows the mean ratios and significant differences within the sites. One-way ANOVA showed no significant differences among the genotypes.

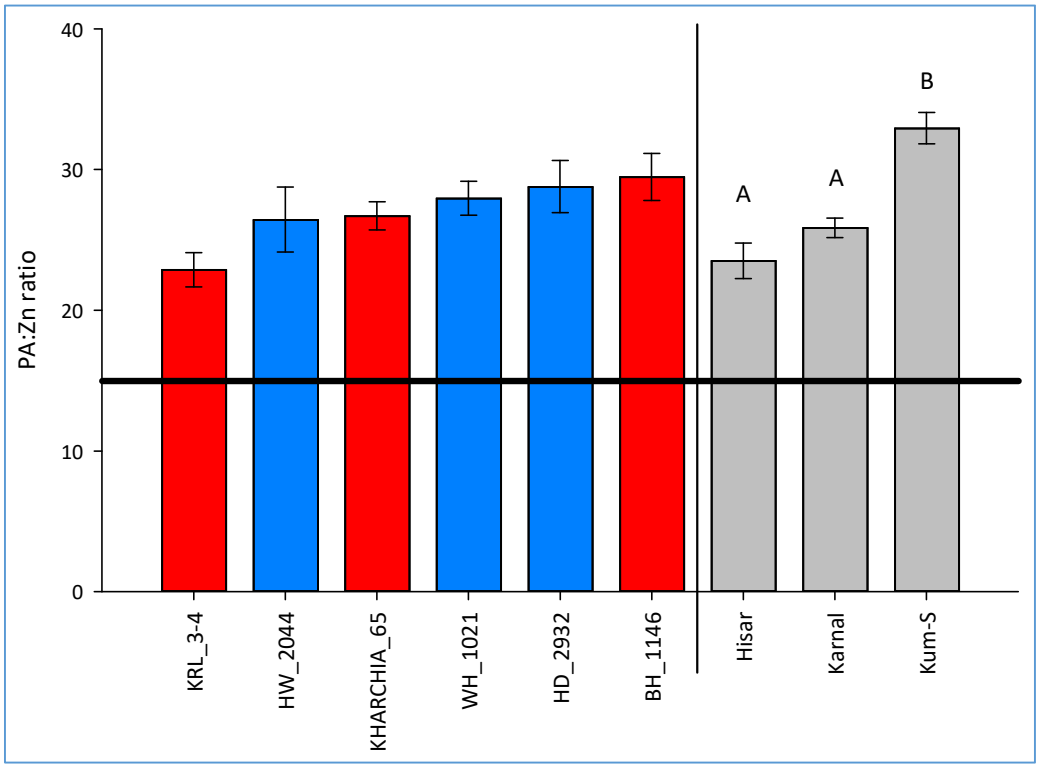



Figure 4-11 One-way ANOVA of PA:Zn ratios. Means ± SE bars. Left side of the vertical line are ratio means by genotype averaged across the three sites. Red bars, HighP-HighZn genotype; blue bars, LowP-LowZn genotype. Genotypes are sorted from lowest to highest mean. Right side of the vertical line are the ratio means by site averaged across the six genotypes. Means followed by a common uppercase letter are not significantly different by Fisher's protected LDS test at the p=0.05 level of significance. Sites are sorted from lowest to highest mean. Horizontal line at y=15 represents the critical value for PA:Fe ratio. Kum-S, Kumarganj sodic site.

For PA x Ca:Zn ratios, site (LSD 1 %=23.18) and genotype x site interaction (LSD 1 %=56.63) were significant ($p<0.001$) while the genotype had no significant effect ($p=0.438$). The overall mean was 264.75 with a range of 192.36 ± 13.43 to 339.95 ± 16.89. Figure 4-12 shows the mean ratios and significant differences within the sites.

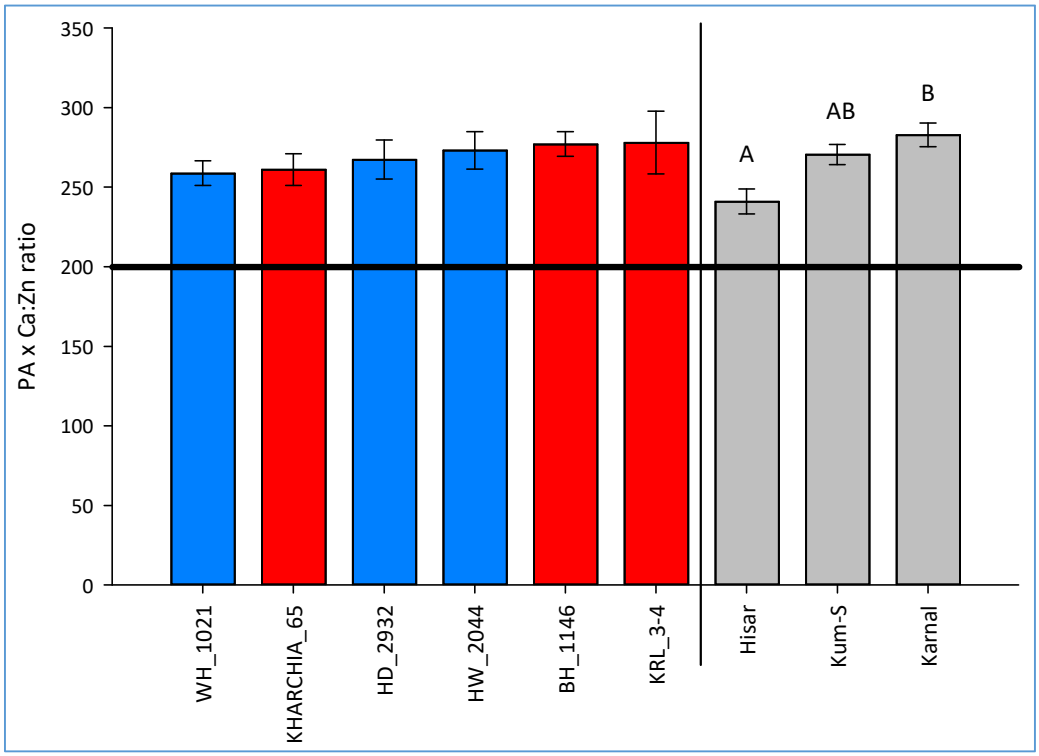



Figure 4-12 One-way ANOVA of PA x Ca:Zn ratios. Means ± SE bars. Left side of the vertical line are ratio means by genotype averaged across the three sites. Red bars, HighP-HighZn genotype; blue bars, LowP-LowZn genotype. Genotypes are sorted from lowest to highest mean. Right side of the vertical line are the ratio means by site averaged across the six genotypes. Means followed by a common uppercase letter are not significantly different by Fisher's protected LDS test at the p=0.05 level of significance. Sites are sorted from lowest to highest mean. Horizontal line at y=200 represents the critical value for PA x Ca:Zn ratio. Kum-S, Kumarganj sodic site.

Contributions of G, E and G x E interaction are shown in Table 4-12. The contributions varied for each one of the ratios. PA:Ca had most of the variance accounted by the genotype (35 %) although followed closely by the environment effect (32 %). In contrast, PA:Fe and PA x Ca:Zn had most of the variance explained by the G x E interaction (53 % and 43 %, respectively). In PA:Zn the greatest amount

of variance was explained by the environment (38 %). PA x Ca:Zn was the only one where the genotype was not significant and where the residual accounted for more than a third of the total (residual = 36 %).

Table 4-12 Effects of genotype, environment and their interaction (G x E) expressed as % of the total sum of squares from ANOVA analysis for phytic acid in wholegrain flour of six genotypes grown at three different sites in India in 2013/14.

| | G | p | E | p | G x E | p | Residual |
|---|---|---|---|---|---|---|---|
| **PA:Ca** | 35.0 | <0.001 | 32.4 | <0.001 | 24.4 | <0.001 | 8.2 |
| **PA:Fe** | 20.4 | <0.001 | 10.3 | <0.001 | 52.7 | <0.001 | 16.7 |
| **PA:Zn** | 13.9 | <0.001 | 37.9 | <0.001 | 29.9 | <0.001 | 18.3 |
| **PA x Ca:Zn** | 3.3 | 0.438 | 17.2 | <0.001 | 43.3 | <0.001 | 36.2 |

df, degrees of freedom; SS, sum of squares; SS (%), SS as percentage to the total; MS, mean squares; p, probability; SE, standard error; Adj. $R^2$, adjusted $R^2$ (%); CV, coefficient of variation (%); Environment = Site.

## 4.5 DISCUSSION AND CONCLUSIONS

In this study, we evaluated the PA concentrations in a subset of six elite genotypes of wheat *Triticum aestivum* L. grown at three different sites, two hostile and one normal, in India. Genotypes were selected based on the concentrations of P and Zn in wholegrain. Three replicates per genotype at the hostile soils and six replicates per genotype at the normal soil were analysed making a total of 72 phytic acid determinations.

The global PA mean in our data set was 8372 ± 1978 mg $kg^{-1}$ (mean ± SD) with a range of 4307 to 11963 mg $kg^{-1}$, and a median value of 8515 mg $kg^{-1}$.

Taking into account that Zn concentrations lower than 15-20 mg $kg^{-1}$ in leaves is considered that the plant is Zn deficient (Jin S. B., 1996 cited by Wang (2015)). Our plants in the hydroponic system from the P experiment treatment 3 (P=0.25 mmol $L^{-1}$, Zn=1 µmol $L^{-1}$) with a mean Zn concentration of 17.06 mg $kg^{-1}$ were Zn deficient and equally the plants in the Zn experiment treatments 1 and 2 (Zn= 0.1 and 1 µmol $L^{-1}$, P=0.25 mmol $L^{-1}$) with a mean Zn concentration of 13.47 and 16.55 mg $kg^{-1}$.

The mean value obtained for the Indian set was 40 % less than the wheat in phosphorus treatment 3 and 37 % less than the plants in Zn treatments 1 and 2.

Our results are comparable to those obtained in advanced breeding lines grown in India (Shitre et al., 2015), 4970 to 15020 mg $kg^{-1}$, mean of 9580 mg $kg^{-1}$, however our highest value was lower. The same author supports the idea that this big range

of genetic variability makes it feasible to develop wheat genotypes with lower phytate content. Other previous findings, include those of Erdal (2002) who reported PA ranges of 7000-12000 and 8000-13000 mg $kg^{-1}$ in wheat cultivars grown in Zn deficient calcareous soils in Central Anatolia with and without Zn, respectively. The authors demonstrated that Zn fertilization resulted in decreases in PA and increases in Zn concentrations and consequently the phytate ratios in seeds decreased on average 126 to 56. Yenagi & Basarkar (2008) reported PA values much higher than ours in wholegrain cereals of North Karnataka, India: bread wheat, PA=23900 mg $kg^{-1}$; durum wheat, 19300 mg $kg^{-1}$; dicoccum wheat 19000, mg $kg^{-1}$. Qazi (2003) reported similar values to our mean in wholegrain flours of Pakistan. In contrast Sachdeva (2013) obtained a range of PA of 2067.1 to 2401 mg $kg^{-1}$ of PA in unprocessed wheat varieties, which is around 50 % our minimum value, such extreme differences might be caused by a sum of variables like the method used to determine the PA, inherent genetic variation and/or soil management (Nitika & Khetarpaul, 2008).

Across the three sites, the genotype KHARCHIA_65 had the highest content of phytic acid (10195.8 mg $kg^{-1}$) meanwhile the lowest concentration was found in genotype HW_2044 (6999.62 mg $kg^{-1}$). Across all the genotypes, the highest PA mean concentration was found at Kumarganj-sodic, 9168 mg $kg^{-1}$ and the lowest at Hisar, 7365 mg $kg^{-1}$. Compared to the normal site, Karnal (8478 ± 1720 mg $kg^{-1}$), in Kumarganj-sodic site the samples had 8 % more PA and in Hisar samples had 13 % less PA.

Genotype, site and genotype x site interaction were statistically significant ($p<0.001$) for PA concentrations in wholegrain. Khan (2007) also observed genotype, environment and interaction significant for PA in wheat grown in Pakistan, PA concentrations varied between 9800-21700 mg $kg^{-1}$, the authors found that the main source of variation was the environment, in this study the main source of variation was the genotype (43.9 %) followed by the interaction (29.4 % of the total sum of squares). Genotype KHARCHIA_65 and BH_1146 had significantly higher levels of PA compared to the other four genotypes.

Previous studies have highlighted the importance of the yield dilution effect (McDonald et al., 2008). It has been observed that in some studies with diverse germplasm there are usually significant variations in the grain yield and it has an impact on the grain nutrient concentrations, for example, high grain Zn concentration has been negatively correlated with the grain yield. Consequently measuring nutrients without taking the grain yield into consideration may result in wrong conclusions. Because we observed big differences in grain yield in this sub-set of genotypes we decided to examine any correlations of the minerals analysed with the grain yield. We found PA, Fe, P and Zn negatively correlated with the grain yield.

Data was corrected and the results were expressed as kg $ha^{-1}$. The results indicated that genotype, site and the interaction genotype x site were significant ($p<0.001$) however, the genotypes with the lowest and highest concentrations were

different. Additionally the main source of variation shifted to the environment (48 %) followed by the genotype effect (35 %).

Pooled data of sites revealed that the genotype KRL_3-4 had the lowest PA concentrations (12.8 kg $ha^{-1}$) followed by KARCHIA_65 (15.9 kg $ha^{-1}$). The highest concentration corresponded to genotype WH_1021 (33.95 kg $ha^{-1}$). This makes evident that there was a grain dilution. Given this differences it is recommended that when breeding for PA as well as for any other mineral, the impact of the grain yield should not be overlooked.

Phosphorus-Zinc status was associated with the levels of PA significantly; the genotypes in the group HighP-HighZn had 30 % less PA than the genotypes in the group LowP-LowZn. An explanation for this could be that under Zn deficiency plants tend to activate genes for P uptake.

Phytic acid is the main storage form of phosphorus for the plant, 60-80 % of the phosphorus is stored as PA, and hence phosphorus could be a good indicator of phytic acid concentrations.

However, P-Zn interactions are complex, there are some evidence indicating that Zn deficiency may be associated with an increase in P uptake/or tissue P concentration. Huang (2000) found in barley that under Zn deficiency, the tight control of P uptake is lost and it leads to very high accumulation of P in plants. The authors described that the Zn deficiency induced the expression of high-affinity phosphate transporter genes. Zhu (2001) reported that Zn had little effect on P

tissue concentration and P uptake and that an increase in P availability caused a reduction in Zn uptake and tissue concentration in wheat plants. The antagonistic effect between P and Zn has been demonstrated, for example, Yang (2011) reported Zn supply decreased the concentration of PA, the authors concluded that an excess of P decreased Zn distribution in grain whereas Zn enhanced the uptake of Zn and P in grain. P and Zn interactions have been widely reported (Parker et al., 1992; R. Shi et al., 2008; Snehi Dwivedi et al., 1975).

In our subset of samples there were no differences on P or Zn concentrations in grain between the sites, however on the full set of samples (Khokhar et al., 2018) did observed differences in Zn concentrations between sites and also reported the soil absorbable Zn was very low.

All three sites were significantly different from each other. The normal site, Karnal, had the highest PA levels, 52.7 % higher compared to Hisar and 172.7 % higher compared to Kumarganj-sodic site. This could be explained by the availability of the nutrients in the soil. It is expected that nutrients are more available for wheat at a pH 6.0-7.0. Plants can undergo a process called luxury consumption when there are excess of nutrients (Horst Marschner, 2002; Winkler & Zotz, 2009), this could explain why the normal site had the greatest concentrations of PA.

Plant roots take up P from the soil solution as orthophosphate ions, principally $H_2PO_4^-$ and to a lesser extent $HPO_4^{2-}$, except in calcareous and saline soils (Syers et al., 2013). The sites where the wheat was grown varied considerably; Karnal soils

were pH 7.5, with an EC of 0.2, dS $m^{-1}$, and organic carbon (OC) content of 0.45%. In contrast, Hisar soils were pH 8.1, with an EC of 0.41 dS $m^{-1}$ and OC of 0.58%. Kumarganj-sodic site soils were pH 9.4, with an EC of 0.7, dS $m^{-1}$ and OC of 0.34% (Khokhar et al., 2017). Hisar and Kumarganj-sodic are classified as saline and sodic, respectively, although the EC reported is much lower than the "required" EC of 4 dS $m^{-1}$ for a soil to be classified as saline, perhaps other parameters were not included in the report. White (2009), reported EC (1:5, soil:water) above 0.21 dS $m^{-1}$ and above 0.4 dS $m^{-1}$ represents a problem in subsoil.

Sodic soils are those which have an exchangeable sodium percentage (ESP) of more than 15. Excess exchangeable sodium has an adverse effect on the physical and nutritional properties of the soil, with consequent reduction in crop growth, significantly or entirely. The EC of saturation soil extracts are, therefore, likely to be variable but are often less than 4 dS/m at 25 °C. Sodic soils have several problems such as lack of aeration and low availability of essential nutrients such as P, Fe, Mn and Zn (Massoud et al., 1988). However some crops have evolved mechanisms to cope with salinity, sodicity and lack of nutrients (Horst Marschner, 2002; Massoud et al., 1988).

We found significant correlations between PA and minerals such as P, Zn, and Fe. This kind of correlations have been previously reported. Phytate is the central molecule in a storage structure in plant cells called the globoid that stores a number of elements besides phosphorous, and is formed in a highly regulated manner and broken down by phytase during germination (Madsen & Brinch-

Pedersen, 2020). Hence, an increase of PA could lead sometimes to more Zn and Fe accumulations in grain, although the exact mechanisms are not fully understood yet. Purified globoids from wheat contained 40 % phytate, 46% protein, 10% moisture and the metallic elements K (7.6%), Mg (3.2%) and Ca (0.43%). Zn, Fe, Cu, Mn and Na were present at <0.1 %. In addition to phosphorous and metallic elements, wheat globoids contained sulphur (0.17%) and trace amounts of boron (1.3 ppm) (L. Bohn et al., 2007)

The strength and significance of the correlations varied between the sites, we observed for example that in Hisar and Kumarganj-sodic a strong negative correlation between PA and calcium. An explanation could be that at these sites calcium was abundant and inhibited P availability and in consequence the PA levels were lower than in the normal site. However more analysis of soil nutrient data should be performed to confirm this.

The strong correlation observed at all sites between P and Zn might be partly explained by our selection of High and Low P-Zn genotypes. Although relationships of this kind have been previously reported, it would be interesting to see these relationships in the whole set of 36 genotypes.

Additionally, it is worth noting that these correlation analysis were performed with data without taking into account the grain yield dilution effect. Perhaps an analysis with partial correlations would be more useful and/or the analysis of a bigger set of genotypes could in part reduce the impact of the GYD.

On average, 67.2 % of the total P was found as phytic acid (phytic acid-P/total P, %). Which is in agreement with previous reports for wheat (Eeckhout & De Paepe, 1994; Steiner et al., 2007; Viveros et al., 2000).

In this study the genotype KRL_3-4 was the genotype with the lowest proportion of P as phytic acid (59.02 %) while the highest one was BH_1146 (76.36 %) which accounts for a 29.4 % increase. There were significant differences between Hisar and Kumarganj-sodic which could be explained by how the plant reacts and stores phosphorus in hostile soils. Lack of P in saline soils could be an explanation for the low levels of PA in Hisar and the opposite in sodic sites where most of the time, P availability is not a problem (Massoud et al., 1988). An interesting observation is that Kumarganj-sodic site had the lowest levels of PA (taking the GYD into consideration), but also had the highest phytic acid-P/total P percentages, this perhaps could be a response to stress in which the plant translocates all the P as PA in grain.

KRL_3-4 was reported (Khokhar et al., 2017) as a good performer in saline soils and had the lowest levels of PA (considering the GYD dilution), KRL_3-4 has a KARCHIA_65 background which had been selected from salt affected areas of Rajasthan, India. KARCHIA_65 was second best for low levels of PA. These genotypes could be a good source of genetic material to introduce in breeding programs for low PA varieties.

Low levels of PA are desirable because it means minerals such as Fe and Zn will be more available to monogastric animals and therefore will provide better nutrition. A lot of effort towards biofortification of wheat and other crops with more Fe and Zn levels has been done, however bad news is that this means little if PA levels are not taken into account. It has been estimated that when PA:Zn ratios are lower than 5, 50 % of the Zn taken can be absorbed. When ratios are between 5-15, 35 % of the Zn is absorbable and when ratios are higher than 15 the absorption rate is only 15 %.

Phytic acid molar ratios of Ca, Fe and Zn were calculated and compared to the established critical values. PA:Ca, PA:Fe and PA:Zn ratios were all significantly affected by the genotype, site and the interaction genotype x site. Additionally the parameter PAxCa:Zn was calculated, as it is thought to provide a better estimation to the bioavailability of Zn than the PA:Zn alone. We observed great variability in all the ratios (CV %); PA:Ca, 32.5 %; PA:Fe, 23.2 %; PA:Zn, 18.9 % and PAxCa:Zn, 15.4 %.

PA:Ca ratios had significant differences between the high/low P-Zn status, there were higher PA:Ca ratios on the HighP-HighZn group of genotypes and this was not observed for any other mineral. This could be because of the way P and Ca interact at the soil level. Mean ratios per genotype per site ranged between 0.63-2.30 for PA:Ca; 8.7-23 for PA:Fe; 17.1-39 for PA:Zn and 192.4-340 for PAxCa:Zn. Only KRL_3-4 when grown at Hisar is predicted to have a good availability for Zn (PAxCa:Zn ratio = 192.4).

The contribution of G, E and G x E interaction varied for each ratio, for PA:Ca genotype accounted for most of the variation (35 %), for PA:Fe and PAxCa:Zn the interaction G x E accounted for most of the variation (52.7 and 43.3 %, respectively), while in PA:Zn, the environment was the dominant (38 %).

It is evident that all the genotypes studied here were above the recommended values for good mineral bioavailability. PA:Ca ratios are above the critical <0.24 value, PA:Fe are also way above the <1 critical value. PA:Zn although not as high as other studies have reported and some genotypes are close to the <15 critical value, Zn bioavailability would still be compromised, values given by the PAxCa:Zn also reflect the compromised bioavailability of Zn. There seem to be a tendency for the lowest ratios to be from genotypes grown in Hisar, perhaps the soil was P deficient and a case of Zn-P antagonism was present, therefore making the plant more able to absorb Zn and by that decreasing the phytate:Zn ratios.

In the previous study by Khokhar (2018) the genotype KARCHIA_65 had the greatest zinc concentration (34.3 mg $kg^{-1}$) in this study the PA:Zn of this genotype was 26.7, ranking 3$^{rd}$ lowest, HW_2044 had the lowest Zn concentration (26.5 mg $kg^{-1}$) and the PA:Zn ratio was 26.4, ranking 2$^{nd}$ lowest in our subset. As we can see, there are big differences in terms of Zn content but in terms of PA:ratio they are no different from each other. KRL_3-4 ranked highest in PAxCa:Zn (278.1) meaning Zn bioavailability would be compromised. KRL_3-4 had the greatest concentration of Ca (437 mg $kg^{-1}$), in this study ranked as the lowest PA:Ca, 1.01. KHARCHIA_65 and KRL_3-4 genotypes had the greatest grain Fe concentration 42.9 and 47.6 mg

$kg^{-1}$ respectively, in our study the PA:Fe was 13.6 and 18.3 respectively, with KRL_3-4 ranking lowest and KARCHIA_65 ranking second highest. In contrast, HW_2044 had the lowest Fe concentration (26.7 mg $kg^{-1}$) and had a PA:Fe of 15.2 ranking 2$^{nd}$ lowest in our subset.

It is prudent that more genotypes from the initial panel of 36 genotypes are analysed for PA concentrations, however here we showed that genotypes adapted to hostile conditions could be a good source of material to breed for low phytic acid varieties.

# 5. GENERAL DISCUSSION

Phytic acid is the main form of storage of P in seeds, in cereals it accounts for 65-85 % of the total P. Why plants accumulate such high amounts of phosphorus is still unknown, although it is believed it is a trait from primitive ancestors as a coping mechanism and to provide nutrients for the germinating plant. Even though P is an essential nutrient and is needed for major functions in the plant, it has been observed that good plant vigour and/or viability can be achieved with much less phosphorus in the seed (Pariasca-Tanaka et al., 2015; F. Wang et al., 2016).

The search for low phytic acid varieties has been somewhat neglected because a low PA could carry negative effects, which are often negatively overstated and some studies have proved that these are not always the case. The loss of grain yield seems to be inevitable, but putting it into perspective, some yield loss might only be a small price to pay compared to the benefits we could potentially obtain like more sustainable agriculture and better human nutrition.

In this study we compared the three sets of data analysed in the present work. To make comparisons fair, only treatments representing the standard P and Zn in the hydroponics solution were compared here Table 5-1. Taking the global means for comparisons, Paragon in the NFT hydroponics experiment had the greatest amount of PA, 60 % more than the genotypes grown in India and 44 % more than the genotypes derived from the Watkins collection, which have Paragon as one of the parents. All three sets were significantly different ($p<0.001$), the set in this case

explained 36 % of the variation in PA (% total sum of squares from one-way ANOVA). Compared to Paragon all the genotypes had significantly lower median PA values (Mann-Whitney U (Wilcoxon rank-sum) test, $p<0.001$); PxW811 – 10, $p=0.008$; PxW398 – 18 and PxW546 – 25, $p=0.014$; PxW223 – 80 and PxW291 – 39, $p=0.026$. These five last genotypes were those of which only data of one harvest year was available (n=3, instead of 12). Our lowest PA median was the genotype HW_2044 (6877.3 mg $kg^{-1}$) and the second highest was the genotype PxW7 – 2 (10683.1 mg $kg^{-1}$). Assuming normality (based on having a large number of samples) the genotype effect accounted for 51 % (of the total sum of squares).

Regarding the sites, we found the PA mean concentrations in wholegrain flour grown in the glasshouse (hydroponic experiment) were significantly higher compared to the other sites ($p<0.001$), compared to Hisar (hostile soil in India) which had the lowest values. Hisar had 45 % less PA compared to the glasshouse, Figure 5-1. An explanation for this is that in the hydroponic system, the nutrients are fully available for the plant, because the P does not become “fixed” to the substrate which is one of the problems in saline and sodic areas like the hostile soils in India.

Additionally, previous studies have reported that the concentration of phytic acid in seeds is highly dependent on the rate of root uptake of P and its translocation from leaves into seeds (Peck et al., 1980; Raboy & Dickinson, 1984, 1993). Root uptake and shoot accumulation of P are greatly affected by Zn deficiency (Ismail Cakmak & Marschner, 1986; Loneragan et al., 1979; Rengel & Graham, 1995) and

it has been suggested that varied Zn supply can influence phytic acid concentration of seeds of plants grown under Zn- deficient conditions. In barley roots it has been seen that Zn deficiency promotes the activation of P transporter genes increasing the P uptake/tissue P leading to a decrease in Zn uptake and concomitantly low Zn levels in the plant (Huang et al., 2000), while in contrast other authors found no effect of the Zn supply on the expression of P uptake efficiency (Zhu, 2001).

Table 5-1 Comparative table of phytic acid and phytic acid-P/total P % in the three wheat datasets analysed. Data are in mg $kg^{-1}$.

| | | NFT | Watkins* | Indian |
|---|---|---|---|---|
| **Phytic acid** | **n** | 34 | 242 | 72 |
| | **Mean ± SD** | 13475 ± 2566.7 | 9374 ± 1614.8 | 8372 ± 1978.5 |
| | **Range** | 7106.4 - 17732.9 | 4853 - 13219.5 | 4306.8 - 11962.9 |
| | **SE** | 440.2 | 103.8 | 233.2 |
| | **%CV** | 19.0 | 17.2 | 23.6 |
| | | | | |
| **Phytic acid-P /total P %** | **n** | 33 | 240 | 72 |
| | **Mean ± SD** | 62 ± 11.9 | 65 ± 10.1 | 67 ± 12.8 |
| | **Range** | 36.9 - 99.8 | 35.2 - 102.6 | 40 - 92.5 |
| | **SE** | 2.1 | 0.7 | 1.5 |
| | **%CV** | 19.2 | 15.6 | 19.1 |

SD, standard deviation; SE, standard error of the mean; %CV, coefficient of variability; NFT, nutrient film technique hydroponic experiment. *Wholegrain flour data only.

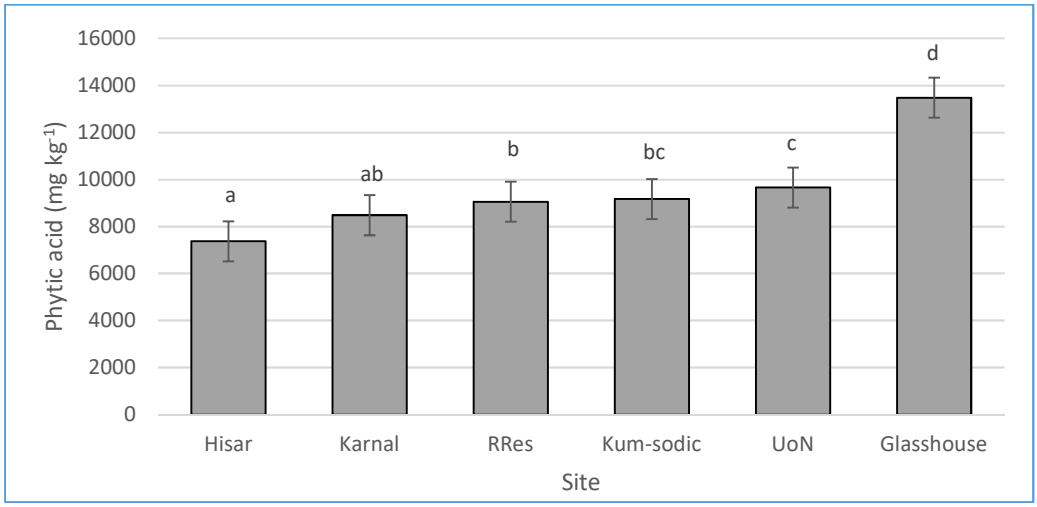



Figure 5-1 Phytic acid concentration in six sites evaluated in this study. Means ± SE bars. Means followed by a common letter are not significantly different by ANOVA and Fisher's protected LDS test at the p=0.01 level of significance. RREs, Rothamsted Research site; Kum-sodic, Kumarganj-sodic site; UoN, University of Nottingham site.

In our study of wheat in hydroponics with variable phosphorus supply affected PA concentrations in wheat leaves and wholegrain, while variable zinc supply had an effect over the PA concentrations in leaves but not in the wholegrain. Phosphorus but not zinc treatments affected grain yield in a hydroponic system. Contrary to previous findings (Yang et al., 2011) in our study P or PA levels did not decrease as a response of Zn increasing supply, but the zinc concentration in grain did increased and by doing so the PA:Zn ratios decreased.

There were no significant differences among our three data sets (Table 5-1) regarding the proportion of P that is found as phytic acid (phytic acid-P/total P %), our global mean values were in agreement with the statement that in mature

grains PA contributes 60-80 % of the total phosphorus (Oberleas, 1973). However, there were some significant differences among the genotypes studied ($p<0.001$), the contribution of the genotype was 17 % of the total sums of squares from ANOVA analysis.

Perhaps one of the most important aspects of this study is the determination of the phytic acid ratios as these are indicative of the potential impact of PA on nutrition Table 5-2. All ratios calculated, PA:Ca, PA:Fe, PA:Zn and PAxCa:Zn ranked significantly higher ($p<0.001$, Kruskal-Wallis One Way Analysis of Variance and Ranks and Dunn's pot hoc test) in the NFT hydroponic experiment than in the Watkins and Indian wheat experiments.

Table 5-2 Comparative table of PA to Ca, Fe, Zn and PA x Ca:Zn ratios in the three datasets analysed.

| | | NFT | Watkins | Indian |
|---|---|---|---|---|
| **PA:Ca** | **n** | **33** | **240** | **72** |
| | **Mean ± SD** | 2 ± 0.5 | 1 ± 0.3 | 1 ± 0.4 |
| | **Range** | 1 - 3.4 | 0.6 - 2.9 | 0.6 - 2.4 |
| | **SE** | 0.1 | 0.0 | 0.0 |
| | **%CV** | 28.1 | 25.5 | 32.5 |
| **PA:Fe** | **Mean ± SD** | 29 ± 8.5 | 23 ± 6.5 | 17 ± 3.8 |
| | **Range** | 14.6 - 56.3 | 12.5 - 43.5 | 8 - 23.7 |
| | **SE** | 1.5 | 0.4 | 0.5 |
| | **%CV** | 28.9 | 27.8 | 23.2 |
| **PA:Zn** | **Mean ± SD** | 66 ± 35.3 | 30 ± 9.5 | 27 ± 5.8 |
| | **Range** | 26.7 - 224.8 | 11.1 - 56.6 | 15.7 - 39.6 |
| | **SE** | 6.1 | 0.6 | 0.7 |
| | **%CV** | 53.1 | 31.1 | 21.4 |
| **PAxCa:Zn** | **Mean ± SD** | 790 ± 276.4 | 338 ± 110.5 | 269 ± 41.5 |
| | **Range** | 323.4 - 1422.5 | 142.3 - 786.6 | 176.9 - 364.6 |
| | **SE** | 48.1 | 7.1 | 4.9 |
| | **%CV** | 35.0 | 32.7 | 15.4 |

PA, phytic acid; SD, standard deviation; SE, standard error of the mean; %CV, coefficient of variability; NFT, nutrient film technique hydroponic experiment.

Some of the genotypes analysed had values significantly lower compared to Paragon, Table 5-3 shows a list of these genotypes.

Table 5-3 Genotypes with PA and PA-ratios lower than Paragon. Kruskal-Wallis One Way Analysis of Variance on Ranks and Dunn's post hoc test with Paragon as the control genotype ($p<0.001$).

| PA | PA:Ca | PA:Fe | PA:Zn | PA x Ca:Zn |
|---|---|---|---|---|
| HD_2932 | HW_2044 | BH_1146 | BH_1146 | BH_1146 |
| HW_2044 | KRL_3-4 | HD_2932 | HD_2932 | HD_2932 |
| KRL_3-4 | PxW264 - 17 | HW_2044 | HW_2044 | HW_2044 |
| PxW264 - 17 | PxW264 - 50 | KHARCHIA_65 | KHARCHIA_65 | KHARCHIA_65 |
| PxW273 - 21 | PxW273 - 21 | KRL_3-4 | KRL_3-4 | KRL_3-4 |
| PxW273 - 71 | PxW273 - 71 | PxW7 - 60 | PxW264 - 17 | PxW254 - 2 |
| PxW291 - 23 | PxW396 - 56 | WH_1021 | PxW273 - 71 | PxW264 - 17 |
| PxW291 - 75 | | | PxW396 - 56 | PxW291 - 23 |
| PxW299 - 87 | | | PxW546 - 20 | PxW396 - 56 |
| PxW396 - 56 | | | PxW546 - 24 | PxW546 - 20 |
| PxW546 - 20 | | | PxW566 - 20 | PxW546 - 24 |
| PxW566 - 20 | | | PxW685 - 36 | PxW566 - 20 |
| PxW7 - 60 | | | PxW7 - 2 | PxW7 - 60 |
| WH_1021 | | | PxW7 - 60 | PxW7 - 76 |
| | | | PxW7 - 76 | PxW811 - 30 |
| | | | PxW811 - 30 | WH_1021 |
| | | | PxW811 - 83 | |
| | | | WH_1021 | |

Comparisons among the sites and the results obtained from the glasshouse experiments, indicated for PA:Ca ratios that there were significant differences in the medians for Rothamsted, Hisar and Karnal ($p<0.001$), UoN ($p=0.047$), indicating there was more calcium at these sites compared to the glasshouse conditions, while there were no significant differences for Kumarganj-sodic site. The lowest mean ratio was observed at Hisar however this value is 4.6-fold the critical ratio of 0.24.

Similar results were observed for PA:Zn ratios, all the sites had lower median ratios than those from the glasshouse ($p<0.001$; except Kumarganj-sodic site, $p=0.001$). The lowest mean ratio observed was at RRes (65 % lower than the glasshouse ratio) but this value was still 55 % higher than the critical ratio of 15.

For PA:Fe median ratios the sites Hisar, Karnal, Kumarganj-sodic and RRes were significantly lower compared to the glasshouse conditions ($p<0.001$), while there was no differences between the glasshouse and UoN site ($p=0.144$). The lowest mean ratio was observed at Hisar but this value represents 15-times the critical ratio of 1.

For PAxCa:Zn median ratios all the sites had lower median ratios than those from the glasshouse ($p<0.001$). The lowest mean ratio observed was at Hisar (2.3-fold lower than the glasshouse ratio) but this value was still 20 % higher than the critical ratio of 200.

In wheat, phytic acid is concentrated in electron-dense parts of the protein storage vacuoles called phytate globoids and these are mainly localized in the aleurone layer. The composition of the globoids has been investigated and has confirmed the connection between these structures and several minerals such as P, Mg, K, Fe and Ca as well as protein and some other minerals but in lower concentrations (L. Bohn et al., 2007). Several reports (De Brier et al., 2016; Joyce et al., 2005; Neal et al., 2013; Regvar et al., 2011) have documented the co-localisation of Zn, Fe, Cu,

Mg, Fe, Na, Al, K, Mn, P in the aleurone layer, all suggesting they are phytate bound.

This supports the strong link between PA and Ca, Mg, Mn, P and Zn that we found in wheat leaves as well as with Cu, Fe, K, Mg, P and Zn in wholegrain flour and also the relationship with the P supply.

Interestingly, varying the Zn supply did not induce the same effects. Wholegrain PA concentrations under controlled Zn supply were only associated significantly with concentrations of P, Cu and Mn. Additionally, under controlled Zn supply, phosphorus concentrations in leaves and PA concentrations in wholegrain were not significantly correlated.

It is known that in wheat the translocation of P to grain occurs mainly from the vegetative tissues before the flowering, after flowering the uptake of P is almost negligible. In this study we provide evidence of the correlation between the leaf P concentrations and the PA concentration in wholegrain in wheat. Therefore, it is possible to estimate the concentration of PA and other minerals such as Mg, Ca, Zn, Mn, Co, Cu and K from the analysis of wheat leaves. This could be of great use in phenotyping studies. Raboy & Dickinson (1993) reported in soybean that variations in seed P is found as variation in phytic acid P, and that this variation is related to leaf P concentration in a complex manner determined by genotype and soil P availability.

The effect of G, E and G x E, varied largely in each set and for each trait analysed, confirming that PA concentration in crops can be widely affected by climatic factors, cultivar differences, fertilizer applications and starting soil conditions. This study provides evidence of the large variability of PA concentrations in diverse environments and genotypes.

## 6. FINAL REMARKS

Efforts have been made to limit PA concentration to minimum (520–4560 mg $kg^{-1}$) in staple food crops (maize, rice and wheat) by employing plant breeding and genetic engineering techniques (Guttieri et al., 2006a). Since PA is considered an antinutrient due to its chelating properties, increasing Pi content in grain while reducing PA has become a trait of interest.

Reducing PA in grains will make food more nutritious, will reduce the P mining of soils and will ameliorate the heavy use of fertilizers that eventually end up in water bodies contributing to pollution. Heavy use of P fertilizers is expensive and unsustainable.

Plants only use an estimated 20-30 % of all the P input. Most of it is sequestered as PA, which cannot be digested by monogastric animals. World reserves of high-quality rock phosphate are expected to be exhausted within the next 80–100 years and it is a non-renewable resource, hence action is necessary to create better crops with better P-efficiency and less antinutrients.

We presented here a set with huge PA variability in different environments. This data could be useful to identify and generate varieties of wheat with lower levels of phytic acid and appropriate levels of micronutrients. Phytic acid as well as mineral traits and their interactions are quite complex but the huge variability observed represents great potential for improvement. We stress the importance

of PA determinations and shout to include them as routine analysis when breeding or searching for mineral enhanced varieties.

## 6.1 FURTHER WORK AND RECOMMENDATIONS

- Some variables did not follow a normal distribution; in some cases, the non-parametric analysis was done but not all were performed.

- More work on correlation analysis and linear regressions is pending. Testing for the individual effect of each treatment on the concentrations of different minerals could provide a larger knowledge of mineral interactions in a controlled system. Samples not used for the PA determination are stored in the lab and more tests could be performed.

- The commercial kit used for the PA determination, is based on the fact that in unprocessed grains the majority of P is in the form of PA. However, it cannot distinguish between lower phosphorylated inositol phosphates, other analytical methods such as HPLC are necessary to differentiate these forms. Because only IP6 and IP5 are known to cause disruptions on the bioavailability of minerals, it would be useful for example to know the full profile of the genotypes in the Watkins diversity set and of those varieties grown in hostile soils.

- It remains to be discussed if the GYD data correction is necessary for the correct analysis of these samples. For example for the Watkins dataset, GYD for the second year was not available.

- Analysing the full set of genotypes of the Indian wheat would provide with a better picture of the PA interactions.
- Soil mineral data is necessary to establish the connection to PA and other mineral concentrations in seed. Valuable information could arise from these analysis in the Watkins data set and in the Indian wheat dataset.
- In this study inorganic P (Pi) was not analysed, it is recommended to add this determination to get a more complete profile of phosphorus.

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# 9. APPENDICES

## 9.1 APPENDIX 1

### 9.1.1 Plant details and identification numbers used in the NFT experiments

Table 9-1 Plant identification numbers.

| Set | Experiment | Bench | Treatment | Plant ID number |
|---|---|---|---|---|
| **One** | Phosphorus | A | 1 | 1-10 |
| | | | 2 | 11-20 |
| | | | 3 | 31-40 |
| | | | 4 | 21-30 |
| | | B | 1 | 61-70 |
| | | | 2 | 71-80 |
| | | | 3 | 51-60 |
| | | | 4 | 41-50 |
| | Zinc | A | 1 | 81-90 |
| | | | 2 | 91-100 |
| | | | 3 | 111-120 |
| | | | 4 | 101-110 |
| | | B | 1 | 141-150 |
| | | | 2 | 151-160 |
| | | | 3 | 131-140 |
| | | | 4 | 121-130 |
| **Two** | Phosphorus | A | 1 | 161-170 |
| | | | 2 | 171-180 |
| | | | 3 | 191-200 |
| | | | 4 | 181-190 |
| | | B | 1 | 221-230 |
| | | | 2 | 231-240 |
| | | | 3 | 211-220 |
| | | | 4 | 201-210 |
| | Zinc | A | 1 | 241-250 |
| | | | 2 | 251-260 |
| | | | 3 | 271-280 |
| | | | 4 | 261-270 |
| | | B | 1 | 301-310 |
| | | | 2 | 311-320 |
| | | | 3 | 291-300 |
| | | | 4 | 281-290 |
| **Three** | Phosphorus | A | 1 | 321-330 |
| | | | 2 | 331-340 |
| | | | 3 | 351-360 |

| Set | Experiment | Bench | Treatment | Plant ID number |
|---|---|---|---|---|
| | | | 4 | 341-350 |
| | | B | 1 | 381-390 |
| | | | 2 | 391-400 |
| | | | 3 | 371-380 |
| | | | 4 | 361-370 |
| | Zinc | A | 1 | 401-410 |
| | | | 2 | 411-420 |
| | | | 3 | 431-440 |
| | | | 4 | 421-430 |
| | | B | 1 | 461-470 |
| | | | 2 | 471-480 |
| | | | 3 | 451-460 |
| | | | 4 | 441-450 |

### 9.1.2 Plant growth in the NFT hydroponic system

Table 9-2 Plant growth details.

| **Key dates phosphorus glasshouse experiment Set One** | |
|---|---|
| Date | Notes |
| 20 June 2016 | Sowing day, Paragon seeds coated with Fludioxonil. Day 1. |
| 22 June 2016 | 147 seeds have germinated. Day 3. |
| 27 June 2016 | 100 % germination. Plantules put in vernalisation period in cold room. Temperature 6 °C day, 8 °C. 12 h photoperiod. Day 8. |
| 27 July 2016 | Vernalisation period finished. Day 38. |
| 1 August 2016 | Plantules out of the cold room and put in glasshouse E. Fed with Hortimix Std 17-7-30 whilst the NFT is being set up. |
| 17 August 2016 | 1st attempt to transplant into NFT. Soil sampling. Leaf sampling and oven dried. GS 13. Some plants were saved in case something happens. Day 59. |
| 24 August 2016 | Nutrient solution reformulated. Last one was too concentrated, salts kept precipitating. Spare plants were transferred into the NFT. Nutrient solution sampling. Day 66. |
| 31 August 2016 | 2nd nutrient solution change. Sampling solution before and after change. Day 73. |
| 6 September 2016 | Soil with roots air dried for analysis. Day 79. |
| 7 September 2016 | 3nd nutrient solution change. Solution sampling, kept at -20 °C. Day 80. |
| 13 September 2016 | Compost sieves 2 mm, after root removal. Day 86. |
| 14 September 2016 | Nutrient solution cancelled due to glasshouse spraying. Day 87. |
| 15 September 2016 | 4th nutrient solution change. T1 and 4 analysed (24.08.2016) for multielemental to see if the concentrations administered are correct. Day 88. |
| 21 September 2016 | 5th nutrient solution change. Water and leaf sampling. Day 94. |
| 28 September 2016 | 6th nutrient solution change. Water and leaf sampling. Day 101. |
| 5 October 2016 | 7th nutrient solution change. Lights turned on slightly in the glasshouse. Leaf sampling for ICP-MS multielemental analysis. Day 108. |
| 12 October 2016 | 8th nutrient solution change. Glasshouse settings change. Day 115. |
| 18 October 2016 | Glasshouse settings change: day temp 24 °C venting at 25 °C. Night temp 18 °C venting at 19 °C. Day length 18 h; 05:00-23:00. Day 121. |
| 19 October 2016 | Nitrogen reduced by ¼. $NH_4NO_3$ (2 mmol L-1 to 1.5 mmol L-1). Noticed Cu deficiency. Low water levels detected mostly in T3 bench A and T4 bench B (Might be because of the lamps). A step was put under the nutrient container to keep the water pump submerged. Day 122. |
| 24 October 2016 | Power failure. Plants left without water flow for one day. Most affected were in bench B, T3 and 4. Probably because they were bigger plants and thus transpiring a lot more than the rest. Day 137. |
| 26 October 2016 | Copper was corrected in the solution. I was feeding them with 1 µM CuSO4 and the recipe had a note saying it should be 3 µM. Day 129. |
| 1 November 2016 | Nutrient solution change. Day 135. |

| Key dates phosphorus glasshouse experiment Set One | |
|---|---|
| Date | Notes |
| 9 November 2016 | Nitrogen reduced ($NH_4NO_3$ 1.5 mmol L-1 to 1.0 mmol L-1). Day 143. |
| 17 November 2016 | Nutrient solution change and sampling. Day 151. |
| 22 November 2016 | 1st ear harvested (although some plants were not ready). Day 157. |
| 24 November 2016 | Last nutrient solution change. Day 159. |
| 1 December 2016 | Water sampling. Leaf samples were put in the freeze dryer and left over the weekend. Most plants have filled grain. Day 166. |
| 5 December 2016 | Water sampling. Tanks filled up with RO water. Drought starts, watering 2 h day, 6 h night. 06:01 -11:59 on; 12:00-14:00 off; 14:01-23:59 on; 00:00-06:00 off. |
| 6 December 2016 | Leaves taken out the freeze dryer. |
| 7 December 2016 | Drought settings changed to: 2 min day, 2 min late afternoon. 06:00 -06:02 on; 06:03-17:59 off; 18:00-18:02 on; 18:03-05:5900 off. |
| 13 December 2016 | Glasshouse temperature settings: 20 °C day, 17 °C night. Water flow switched off, plants left off to mature. |
| 11 January 2016 | Rest of ears harvested and left in paper bags in the glasshouse to dry off any residual moist. Ears were dehusked manually (rubbing). |

| Key dates zinc glasshouse experiment Set One | |
|---|---|
| Date | Notes |
| 19 September 2017 | 150 seeds sown in rockwool plugs. Left in glasshouse E4. Watered with RO water from the glasshouse. Day 1. |
| 25 September 2017 | Approximately 100 seeds have germinated. |
| 26 September 2017 | Seedlings were transferred to the cold growth room A09. Room conditions are 6 °C day/night 12 h photoperiod. Feeding with half strength Hoagland's solution (one litre per week approximately). |
| 29 September 2017 | Tray filled with 1 L half strength Hoagland's solution. Also on the 3rd and 9th October. |
| 3 November 2017 | Plants were taken out the cold room and left in the glasshouse for a few days until transplanted into the NFT system. Glasshouse lamps on. Temperature 22 °C day, 18 °C night. |
| 7 November 2017 | Plants transferred to into the NFT system. Most of them are in GS 14 (4 leaves unfolded) and some already have tillers (GS 21). Leaves and stems from the unused plants were collected and weighted (fresh weight) and left in the lab cold room (4 °C) in a paper and plastic bag. |
| 14 November 2017 | 2nd nutrient solution change. Plants looking good. Some look lodged. |
| 16 November 2017 | Remaining plant leaves collected and stored like the previous ones. |
| 22, 29, November, 5 December 2017 | Nutrient solution change. |
| 12 December 2017 | Nutrient solution change. 1st spike was labelled on some plants. |
| 30 December 2017 | Nutrient solution change. Plants look good. Some tanks were very low on water but no casualties. |
| 9 January 2018 | Nutrient solution change. $NH_4NO_3$ reduced to 1.5 mmol L-1. Day 113. |
| 16 January 2018 | Nutrient solution change. $NH_4NO_3$ reduced to 1.0 mmol L-1. |

| **Key dates zinc glasshouse experiment Set One** | |
|---|---|
| Date | Notes |
| 23 January 2018 | Made a mistake swapping pipes for treatments 1 and 3. Plants got draught but don't look dead. Grain and heads looking good. Future analysis must be compared to those on bench A to see if they are reliable. Leaf sampling of all plants, leaves 2 and 2 from top of each spike were collected in paper bags. Left in the cold room to be powdered with liquid nitrogen. |
| 25 January 2018 | Nutrient solution change. $NH_4NO_3$ reduced to 0.5 mmol L-1. Plants looking senescent and some heads are fully mature. |
| 1 February 2018 | Timed draught. Plants put in RO water, timer set to water 2 h day, 6 h night. Water samples taken for ICP-MS multielemental analysis. Labelled as water_ZnExp_final_(bench-treatment). |
| 5 February 2018 | Watering was stopped completely. |
| 20 February 2018 | Harvest. Heads were put in paper bags to be processed later. |

| **Key dates P and Zn glasshouse experiment Set Two** | |
|---|---|
| Date | Notes |
| 13 February 2018 | 150 Paragon seeds sown in rockwool propagation trays, watered with RO water. |
| 20 February 2018 | Seeds put in cold room for vernalisation 6 °C day/night, 12 h photoperiod. 4 weeks – 20$^{th}$ feb to 20$^{th}$ march. |
| 21 February 2018 | New batch of Paragon (batch 2) sown to have enough seed for both P and Zn experiments simultaneously. |
| 28 February 2018 | Bach 2 transferred to cold room for vernalisation. |
| 1 March 2018 | Plants sown on the 21$^{st}$ were put in A12 due to an outbreak in A09 (temperature was 17 °C |
| 5 March 2018 | Plantules fed with Hoagland's half strength and put back in A09. |
| 27 March 2018 | Plants taken out the cold room and left in the glasshouse watered with RO water. |
| 28 March 2018 | NFT set up with 160 plants. Out of the 300 sown, 160 were randomly selected and allocated in the system.<br>Batch 1: pretty much all looking good, GS 21 most plants (no tillers yet)<br>Batch 2: most looking ill with yellow and purple tips, notable smaller than Batch1.<br>Something was wrong with the seeds of batch 2, I left them in the heat in the glasshouse. For this reason only the best-looking plants were selected. This was unusual and never happened before. Tanks were filled up to prepare 60 L of solution and then it was split into 30 L for the other bench.<br>Water collected for ICP-MS analysis, filtered with 0.2 µm and diluted 9.6 mL sample + 400 µL $HNO_3$ 50 %. |
| 29 March 2018 | Leaves of the unused plants were collected and oven dried, GS 21 most 3 tillers depending which batch. |
| 4 April 2018 | 2$^{nd}$ nutrient solution change. |
| 11 April 2018 | 3rd nutrient solution change. |
| 18 April 2018 | 4$^{th}$ nutrient solution change. |
| 25 April 2018 | 5$^{th}$ nutrient solution change. |
| 2 May 2018 | 6$^{th}$ nutrient solution change. |
| 7 May 2018 | Tanks were running very low on solution and they were topped up with RO water. |
| 9 May 2018 | 7$^{th}$ nutrient solution change. Solution volume increased to 80 L. T4 P experiment from Bench B were the most affected by drought. Seems like they could not recover 100 %. Also they were notably affected by aphids. |

| Key dates P and Zn glasshouse experiment Set Two | |
|---|---|
| Date | Notes |
| 15 May 2018 | Tanks topped up with 10 L of RO water. |
| 17 May 2018 | 8$^{th}$ nutrient solution change. Leaf sampling, put in the oven 50 °C |
| 19 May 2018 | Leaf sampling, put to dry in the oven. Most plants are in GS 61. |
| 21 May 2018 | Leaf sampling, tanks topped up with RO water because were running low. |
| 22 May 2018 | Leaf sampling |
| 23 May 2018 | 9$^{th}$ nutrient solution change. Nitrogen reduction to 1.5 mmol $L^{-1}$ $Ca(NO_3)_2$, (by mistake, instead $NH_4NO_3$). Leaf sampling. Day 100. |
| 29 May 2018 | 10$^{th}$ nutrient solution change. Nitrogen reduction to 1.0 mmol $L^{-1}$ $Ca(NO_3)_2$. |
| 7 June 2018 | Last nutrient change (11$^{th}$), Nitrogen reduced to 0.5 mmol $L^{-1}$ $Ca(NO_3)_2$. |
| 11 June 2018 | Plants put in RO water (80 L). Timer set 4 h day, 2 h night. |
| 18 June 2018 | Water switched off completely. |
| 4-6 July 2018 | Harvest. Ears were counted. |
| 10-11 July 2018 | Ears dehusked. |
| 8, 9, 11, 14 July 2018 | Leaves milled |
| 16 July 2018 | Grain samples for PA analysis were milled. |
| 19, 20 July 2018 | Microwave digestion of selected samples. PA analysis. |

| Key dates P and Zn glasshouse experiment Set Three | |
|---|---|
| Date | Notes |
| 11 June 2018 | Approximately 300 Paragon seed were put in germination trays and watered with RO water. |
| 18 June 2018 | The two trays were put in A09 for vernalisation period. 6 °C day/night 12 h photoperiod. Feeding with half strength Hoagland's solution (one litre per week approximately). |
| 23 July 2018 | Plants taken out the cold room and left in the glasshouse E. |
| 24 July 2018 | Plants transferred to the NFT system. Start treatments P and Zinc. 160 plants in total: IDs 321-480. |
| 25 July 2018 | T2 in bench B Phosphorus leaking so the whole solution was replaced. |
| 1 August 2018 | Nutrient solution change. Water sampling |
| 7 August 2018 | Nutrient solution change. |
| 15 August 2018 | Nutrient solution change. |
| 22 August 2018 | Nutrient solution change. |
| 29 August 2018 | Nutrient solution change. |
| 30 August 2018 | First leaves sampled. |
| 31 August 2018 | First leaves sampled. |
| 3 September 2018 | Nutrient solution change. Increased to 80 L. |

| **Key dates P and Zn glasshouse experiment Set Three** | |
|---|---|
| Date | Notes |
| 12 September 2018 | Nutrient solution change. |
| 19 September 2018 | Day 100. |
| 20 September 2018 | Nutrient solution change. $NH_4NO_3$ reduced to 1.5 mmol L-1. All plants have been sampled (leaf). Most have already flowered with a few exceptions and quite a lot are filling grain. |
| 27 September 2018 | Nutrient solution change. $NH_4NO_3$ reduced to 1.0 mmol L-1. |
| 3 October 2018 | Last nutrient solution change. $NH_4NO_3$ reduced to 0.5 mmol L-1. |
| 8 October 2018 | Plants put in RO water (30 L), intermittent watering. Timer settings: 4 h day, 2 h night. Timer on at 08:00 -10:00; 16:00-18:00; 00:00-02:00 on. |
| 15 October 2018 | Watering turned off completely. |
| 29 October 2018 | Selected plants to be analysed were milled in AES lab in Gateway using the old little mill. |
| 30 October 2018 | T1 bench A grain harvested. |
| 1 November 2018 | Rest of the wheat was harvested. |

### 9.1.3 Nutrient solution recipe for NFT hydroponic system

Table 9-3 Nutrient solution recipe for phosphorus experiments.

| | Stock solutions | | | Preparation of final solution | |
|---|---|---|---|---|---|
| | ***MACRONUTRIENTS*** | **Stock solution concentration (mol $L^{-1}$)** | **Stock solution ID number** | **Volume needed for 1 L final solution** | **Final concentration (mmol $L^{-1}$)** |
| **Phosphorus treatment 1** | $KH_2PO_4$<br>KOH | 0.0625<br>0.125 | 1.1 | 1 mL of the stock of desired treatment | 0.0625<br>0.125 |
| **Phosphorus treatment 2** | $KH_2PO_4$<br>KOH | 0.125<br>0.25 | 1.2 | | 0.125<br>0.25 |
| **Phosphorus treatment 3** | $KH_2PO_4$<br>KOH | 0.25<br>0.5 | 1.3 | | 0.25<br>0.5 |
| **Phosphorus treatment 4** | $KH_2PO_4$<br>KOH | 0.5<br>1 | 1.4 | | 0.5<br>1 |
| | $MgSO_4.7H_2O$ | 0.75 | 2.1 | 1 mL | 0.75 |
| | $CaCl_2.2H_2O$ | 0.025 | 2.2 | 1 mL | 0.025 |
| | FeNaEDTA | 0.1 | 3 | 1 mL | 0.1 |
| | $Ca(NO_3)2.4H_2O$ | 2 | 4 | 1 mL | 2 |
| | $NH_4NO_3$ | 2 | 5 | 1 mL | 2 |
| **Phosphorus treatment 1** | $K_2SO_4$<br>KOH | 0.233<br>0.93 | 7.1 | 1 mL of the stock of desired treatment | 0.233<br>0.93 |
| **Phosphorus treatment 2** | $K_2SO_4$<br>KOH | 0.201<br>0.8 | 7.2 | | 0.2<br>0.8 |
| **Phosphorus treatment 3** | $K_2SO_4$<br>KOH | 0.133<br>0.53 | 7.3 | | 0.133<br>0.53 |
| **Phosphorus treatment 4** | ----------T4 does not contain K2SO4/KOH---------- | | | Add 1 mL of RO water | ---------- |
| | ***MICRONUTRIENTS*** | See MICRONUTRIENTS table below | 6 | See MICRONUTRIENTS table below | |

Table 9-4 Micronutrient stock solution for recipe used in phosphorus experiments.

| ***MICRONUTRIENTS*** **Stock solution** | **Concentration (mmol $L^{-1}$)** |
|---|---|
| $H_3BO_3$ | 30 |
| $MnSO_4.4H_2O$ | 10 |
| $ZnSO_4.7H_2O$ | 1 |
| $CuSO_4.5H_2O$ | 3 |
| $Na_2MoO_4.2H_2O$ | 0.5 |

| Take 1 mL of this stock and add to the final solution with the macronutrients | **Final concentration (µmol $^{L-}$)** |
|---|---|
| | 30 |
| | 10 |
| | 1 |
| | 3 |
| | 0.5 |

Table 9-5 Treatment concentrations used in Phosphorus experiments.

| **Treatment number** | **$KH_2PO_4$ (mmol $L^{-1}$)** | **Equivalent to mg $L^{-1}$ of phosphorus** |
|---|---|---|
| 1 | 0.0625 | 1.9 |
| 2 | 0.125 | 3.86 |
| 3 | 0.25 | 7.72 |
| 4 | 0.5 | 15.45 |

Table 9-6 Nutrient solution recipe for zinc experiments.

| | Stock solutions | | Preparation of final solution | |
|---|---|---|---|---|
| ***MACRONUTRIENTS*** | **Stock solution concentration (mol $L^{-1}$)** | **Stock solution ID number** | **Volume needed for 1 L final solution** | **Final concentration (mmol $L^{-1}$)** |
| $KH_2PO_4$ | 0.25 | 1 | 1 | 0.25 |
| KOH | 0.5 | | | 0.5 |
| $MgSO_4.7H_2O$ | 0.75 | 2.1 | 1 | 0.75 |
| $CaCl_2.2H_2O$ | 0.025 | 2.2 | 1 | 0.025 |
| FeNaEDTA | 0.1 | 3 | 1 | 0.1 |
| $Ca(NO_3)2.4H_2O$ | 2 | 4 | 1 | 2 |
| $NH_4NO_3$ | 2 | 5 | 1 | 2 |
| ***MICRONUTRIENTS*** | 6 | 6 | 1 | 2 |

Table 9-7 Micronutrient stock solution for recipe used in zinc experiments.

| ***MICRONUTRIENTS*** | **Stock solution concentration (mmol L-1)** | **Volume needed for 1 L final solution** | **Final concentration (µmol L-)** |
|---|---|---|---|
| $H_3BO_3$ | 30 | 1 mL of the desired treatment stock | 30 |
| $MnSO_4.4H_2O$ | 10 | | 10 |
| $ZnSO_4.7H_2O$ | Depending on treatment required, see Zn treatments below | | Depending on treatment required, see Zn treatments below |
| $CuSO_4.5H_2O$ | 3 | | 3 |
| $Na_2MoO_4.2H_2O$ | 0.5 | | 0.5 |

Table 9-8 recipe for zinc experiments.

| ***Zn TREATMENTS*** | **Stock solution concentration (mmol L-1)** | **Stock solution ID number** | **Volume needed for 1 L final solution** | **Final concentration (µmol L-)** |
|---|---|---|---|---|
| $ZnSO_4.7H_2O$ | 0.1 | 6.1 | 1 mL of the desired treatment stock and add to final solution | 0.1 |
| $ZnSO_4.7H_2O$ | 1 | 6.2 | | 1 |
| $ZnSO_4.7H_2O$ | 5 | 6.3 | | 5 |
| $ZnSO_4.7H_2O$ | 10 | 6.4 | | 10 |

Table 9-9 Treatment concentrations used in zinc experiments.

| **Treatment number** | **$ZnSO_4.7H_2O$ (µM)** | **Equivalent to µg $L^{-1}$ of zinc** |
|---|---|---|
| 1 | 0.1 | 6.538 |
| 2 | 1.0 | 65.38 |
| 3 | 5.0 | 176.9 |
| 4 | 10.0 | 653.8 |

### 9.1.4 Recovery percentages of mineral elements analysed with ICP-MS.

Table 9-10 Measured values and recovery percentage of CRM used. Set One NFT experiment.

| Element | Average CRM tomato leaves measured values | Mass Fraction Values for Elements in SRM 1573a | Recovery (%) | Average CRM wheat flour measured values | Mass Fraction Values for Elements in SRM 1567b | Recovery (%) |
|---|---|---|---|---|---|---|
| **Ag** | 0.01 | 0.017 | 72.51 | 0.00 | not available | ND |
| **Al** | 494.75 | 598.000 | 82.73 | 5.05 | 4.40 | 114.70 |
| **As** | 0.13 | 0.112 | 115.55 | 0.00 | 0.0048 | 55.30 |
| **B** | 33.44 | 33.300 | 100.43 | 0.91 | not available | ND |
| **Ba** | 60.65 | 63.000 | 96.28 | 1.30 | not available | ND |
| **Be** | 0.03 | not available | ND | 0.00 | not available | ND |
| **Ca** | 50952.83 | 50500.000 | 100.90 | 188.99 | 191.4 | 98.74 |
| **Cd** | 1.41 | 1.520 | 92.61 | 0.02 | 0.025 | 86.31 |
| **Co** | 0.49 | 0.570 | 86.39 | 0.01 | not available | ND |
| **Cr** | 2.20 | 1.990 | 110.41 | 0.24 | not available | ND |
| **Cs** | 0.05 | 0.053 | 92.70 | 0.00 | not available | ND |
| **Cu** | 3.92 | 4.700 | 83.45 | 1.60 | 2.03 | 78.68 |
| **Fe** | 336.42 | 368.000 | 91.42 | 11.97 | 14.1 | 84.80 |
| **K** | 27996.77 | 27000.000 | 103.69 | 1165.47 | 1325.0 | 87.96 |
| **Li** | 0.55 | not available | ND | 0.06 | not available | ND |
| **Mg** | 10589.88 | 12000.000 | 88.25 | 358.59 | 398.0 | 90.10 |
| **Mn** | 250.48 | 246.000 | 101.82 | 8.39 | 9.00 | 93.26 |
| **Mo** | 0.42 | 0.460 | 91.41 | 0.43 | 0.464 | 92.48 |
| **Na** | 130.01 | 136.000 | 95.60 | 7.47 | 6.71 | 111.28 |
| **Ni** | 1.49 | 1.590 | 93.78 | 0.18 | not available | ND |
| **P** | 2512.25 | 2160.000 | 116.31 | 1272.44 | 1333.0 | 95.46 |
| **Pb** | 0.51 | not available | ND | 0.00 | 0.0104 | -42.52 |
| **Rb** | 14.65 | 14.890 | 98.38 | 0.63 | 0.671 | 94.09 |
| **S** | 10735.86 | 9600.000 | 111.83 | 1386.53 | 1645 | 84.29 |

| Element | Average CRM tomato leaves measured values | Mass Fraction Values for Elements in SRM 1573a | Recovery (%) | Average CRM wheat flour measured values | Mass Fraction Values for Elements in SRM 1567b | Recovery (%) |
|---|---|---|---|---|---|---|
| **Se** | 0.07 | 0.054 | 135.21 | 1.07 | 1.14 | 93.60 |
| **Sr** | 84.49 | 85.000 | 99.40 | 1.02 | not available | ND |
| **Ti** | 14.47 | not available | ND | 0.07 | not available | ND |
| **Tl** | 0.03 | not available | ND | 0.00 | not available | ND |
| **U** | 0.03 | 0.035 | 72.03 | 0.00 | not available | ND |
| **V** | 0.76 | 0.835 | 91.49 | 0.01 | 0.0100 | 90.62 |
| **Zn** | 26.35 | 30.900 | 85.27 | 9.39 | 11.6 | 80.90 |

CRM, Certified Reference Material; SRM, Standard Reference Material; Not available means the SRM does not provide a value for the element; ND, recovery not determined; in red are recovery values out of the reliable range of 80-120 %. Data are in mg $kg^{-1}$. CRM=SRM.

Table 9-11 Measured values and recovery percentage of CRM used. Set Two NFT experiment.

| Element | Average CRM tomato leaves measured values | Mass Fraction Values for Elements in SRM 1573a | Recovery (%) | Average CRM wheat flour measured values | Mass Fraction Values for Elements in SRM 1567b | Recovery (%) |
|---|---|---|---|---|---|---|
| Ag | 0.01 | 0.017 | 75.6 | 0.00 | Not available | ND |
| Al | 239.90 | 598.0 | 40.1 | 2.83 | 4.40 | 64.4 |
| As | 0.12 | 0.112 | 111.0 | 0.00 | 0.0048 | 75.1 |
| B | 30.07 | 33.30 | 90.3 | 0.88 | Not available | ND |
| Ba | 57.17 | 63.0 | 90.7 | 1.17 | Not available | ND |
| Be | 0.02 | Not available | ND | 0.00 | Not available | ND |
| Ca | 48506.50 | 50500.0 | 96.1 | 195.80 | 191.4 | 102.3 |
| Cd | 1.40 | 1.52 | 91.8 | 0.02 | 0.025 | 83.0 |
| Co | 0.47 | 0.570 | 83.2 | 0.01 | Not available | ND |
| Cr | 1.41 | 1.99 | 70.8 | 0.08 | Not available | ND |
| Cs | 0.04 | 0.053 | 68.2 | 0.00 | Not available | ND |
| Cu | 3.98 | 4.70 | 84.7 | 1.76 | 2.03 | 86.9 |
| Fe | 258.07 | 368.0 | 70.1 | 11.40 | 14.1 | 80.8 |
| K | 25630.17 | 27000.00 | 94.9 | 1590.29 | 1325.0 | 120.0 |
| Li | 0.41 | Not available | ND | 0.05 | Not available | ND |
| Mg | 10095.85 | 12000.0 | 84.1 | 448.74 | 398.0 | 112.7 |
| Mn | 225.62 | 246.00 | 91.7 | 8.49 | 9.00 | 94.3 |
| Mo | 0.39 | 0.460 | 85.9 | 0.43 | 0.464 | 92.6 |
| Na | 110.23 | 136.00 | 81.0 | 3.76 | 6.71 | 56.0 |
| Ni | 1.27 | 1.59 | 79.7 | 0.08 | Not available | ND |
| P | 2284.79 | 2160.0 | 105.8 | 1334.42 | 1333.0 | 100.1 |
| Pb | 0.51 | Not available | ND | 0.09 | 0.0104 | 906.9 |
| Rb | 13.84 | 14.89 | 93.0 | 0.66 | 0.671 | 98.2 |
| S | 9563.33 | 9600.00 | 99.6 | 1477.08 | 1645 | 89.8 |
| Se | 0.08 | 0.054 | 144.3 | 1.12 | 1.14 | 98.4 |

| Element | Average CRM tomato leaves measured values | Mass Fraction Values for Elements in SRM 1573a | Recovery (%) | Average CRM wheat flour measured values | Mass Fraction Values for Elements in SRM 1567b | Recovery (%) |
|---|---|---|---|---|---|---|
| Sr | 83.92 | 85.0 | 98.7 | 0.98 | Not available | ND |
| Ti | 10.61 | Not available | ND | 0.04 | Not available | ND |
| Tl | 0.03 | Not available | ND | 0.00 | Not available | ND |
| U | 0.01 | 0.035 | 42.1 | 0.00 | Not available | ND |
| V | 0.44 | 0.835 | 52.2 | 0.01 | 0.0100 | 75.2 |
| Zn | 27.00 | 30.9 | 87.4 | 10.01 | 11.6 | 86.2 |

CRM, Certified Reference Material; SRM, Standard Reference Material; Not available means the SRM does not provide a value for the element; ND, recovery not determined; in red are recovery values out of the reliable range of 80-120 %. Data are in mg $kg^{-1}$. CRM=SRM.

Table 9-12 Measured values and recovery percentage of CRM used. Set Three NFT experiment.

| Element | Average CRM tomato leaves measured values | Mass Fraction Values for Elements in SRM 1573a | Recovery (%) | Average CRM wheat flour measured values | Mass Fraction Values for Elements in SRM 1567b | Recovery (%) |
|---|---|---|---|---|---|---|
| **Ag** | -0.48 | 0.017 | -2835.2 | -0.25 | Not available | ND |
| **Al** | 333.63 | 598.0 | 55.8 | 2.74 | 4.40 | 62.4 |
| **As** | 0.14 | 0.112 | 125.3 | 0.00 | 0.0048 | 85.3 |
| **B** | 27.59 | 33.30 | 82.9 | -0.74 | Not available | ND |
| **Ba** | 60.18 | 63.0 | 95.5 | 1.23 | Not available | ND |
| **Be** | 0.02 | Not available | ND | 0.00 | Not available | ND |
| **Ca** | 48943.09 | 50500.0 | 96.9 | 201.20 | 191.4 | 105.1 |
| **Cd** | 1.45 | 1.52 | 95.4 | 0.02 | 0.025 | 97.5 |
| **Co** | 0.49 | 0.570 | 86.0 | 0.01 | Not available | ND |
| **Cr** | 1.63 | 1.99 | 82.1 | 0.06 | Not available | ND |
| **Cs** | 0.05 | 0.053 | 88.2 | 0.00 | Not available | ND |
| **Cu** | 3.76 | 4.70 | 79.9 | 1.68 | 2.03 | 82.9 |
| **Fe** | 314.58 | 368.0 | 85.5 | 12.25 | 14.1 | 86.8 |
| **K** | 26193.48 | 27000.00 | 97.0 | 1356.53 | 1325.0 | 102.4 |
| **Li** | 0.52 | Not available | ND | 0.06 | Not available | ND |
| **Mg** | 10615.13 | 12000.0 | 88.5 | 385.49 | 398.0 | 96.9 |
| **Mn** | 231.31 | 246.00 | 94.0 | 8.84 | 9.00 | 98.2 |
| **Mo** | 0.18 | 0.460 | 38.6 | 0.33 | 0.464 | 70.8 |
| **Na** | 96.25 | 136.00 | 70.8 | 5.11 | 6.71 | 76.2 |
| **Ni** | 1.40 | 1.59 | 88.1 | 0.17 | Not available | ND |
| **P** | 2174.62 | 2160.0 | 100.7 | 1315.14 | 1333.0 | 98.7 |
| **Pb** | 0.53 | Not available | ND | 0.02 | 0.0104 | 145.5 |
| **Rb** | 14.31 | 14.89 | 96.1 | 0.67 | 0.671 | 100.0 |
| **S** | 9673.24 | 9600.00 | 100.8 | 1556.30 | 1645 | 94.6 |
| **Se** | 0.06 | 0.054 | 104.2 | 1.15 | 1.14 | 100.7 |

| Element | Average CRM tomato leaves measured values | Mass Fraction Values for Elements in SRM 1573a | Recovery (%) | Average CRM wheat flour measured values | Mass Fraction Values for Elements in SRM 1567b | Recovery (%) |
|---|---|---|---|---|---|---|
| **Sr** | 85.96 | 85.0 | 101.1 | 1.03 | Not available | ND |
| **Ti** | 7.89 | Not available | ND | 0.06 | Not available | ND |
| **Tl** | 0.04 | Not available | ND | 0.00 | Not available | ND |
| **U** | 0.01 | 0.035 | 32.7 | 0.00 | Not available | ND |
| **V** | 0.64 | 0.835 | 77.1 | 0.01 | 0.0100 | 77.5 |
| **Zn** | 27.15 | 30.9 | 87.9 | 10.57 | 11.6 | 91.1 |

CRM, Certified Reference Material; Not available means the CRM does not provide a value for the element; ND, recovery not determined; in red are recovery values out of the reliable range of 80-120 %. Data are in mg kg-1. CRM=SRM.

Table 9-13 Number of values (n) for each of the 9 elements selected for statistical analysis by set, treatment and bench.

| Leaf minerals n | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ca | | Co | | Cu | | Fe | | K | | Mg | | Mn | | P | | Zn | |
| **Set** | **Treatment** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** |
| One | Phosphorus | 8 | 8 | 0 | 0 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| One | Zinc | 12 | 11 | 0 | 0 | 12 | 11 | 12 | 11 | 12 | 11 | 12 | 11 | 12 | 11 | 12 | 11 | 12 | 11 |
| Two | Phosphorus | 12 | 15 | 12 | 15 | 12 | 15 | 0 | 0 | 12 | 15 | 12 | 15 | 12 | 15 | 12 | 15 | 12 | 15 |
| Two | Zinc | 11 | 10 | 11 | 10 | 11 | 10 | 0 | 0 | 11 | 10 | 11 | 10 | 11 | 10 | 11 | 10 | 11 | 10 |
| Three | Phosphorus | 12 | 12 | 11 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| Three | Zinc | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |

| Grain minerals n | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ca | | Co | | Cu | | Fe | | K | | Mg | | Mn | | P | | Zn | |
| **Set** | **Treatment** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** |
| One | Phosphorus | 8 | 8 | 0 | 0 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| One | Zinc | 11 | 12 | 0 | 0 | 0 | 0 | 11 | 12 | 11 | 12 | 11 | 12 | 11 | 12 | 11 | 12 | 11 | 12 |
| Two | Phosphorus | 12 | 15 | 12 | 15 | 12 | 15 | 12 | 15 | 12 | 15 | 12 | 15 | 12 | 15 | 12 | 15 | 12 | 15 |
| Two | Zinc | 11 | 10 | 11 | 9 | 11 | 10 | 11 | 10 | 11 | 10 | 11 | 10 | 11 | 10 | 11 | 10 | 11 | 10 |
| Three | Phosphorus | 13 | 12 | 13 | 12 | 13 | 12 | 13 | 12 | 13 | 12 | 13 | 12 | 13 | 12 | 13 | 12 | 13 | 12 |
| Three | Zinc | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |

### 9.1.5 LOQ , LOD and blank values for NFT experiments by set.

Table 9-14 LOQ, LOD and blank calculations for NFT experiments.

| Set | Run | Sample identifier | Free Phosphorus | Total Phosphorus | Sample weight (g) | Extraction volume (mL) | delta-Abs (Phosphorus) | Total P* (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| One | PA_BLANK_NFTrun1 | 06.09.2017_BLANK | 0.0350 | 0.0617 | 1.0000 | 20.000 | 0.0267 | 174.5652 | 619.0257 |
| | PA_BLANK_NFTrun2 | 07.09.2017_BLANK | 0.0350 | 0.0543 | 1.0000 | 20.000 | 0.0193 | 126.5598 | 448.7936 |
| | PA_BLANK_NFTrun3 | 26.09.2017_BLANK | 0.0350 | 0.0517 | 1.0000 | 20.000 | 0.0167 | 109.1033 | 386.8910 |
| | PA_BLANK_NFTrun4 | 27.09.2017_BLANK | 0.0347 | 0.0567 | 1.0000 | 20.000 | 0.0220 | 144.0163 | 510.6962 |
| | PA_BLANK_NFTrun5 | 11.04.2018_BLANK | 0.0357 | 0.0520 | 1.0000 | 20.000 | 0.0163 | 106.9212 | 379.1532 |
| | PA_BLANK_NFTrun6 | 12.04.2018_BLANK | 0.0360 | 0.0533 | 1.0000 | 20.000 | 0.0173 | 113.4674 | 402.3667 |
| | PA_BLANK_NFTrun7 | 13.04.2018_BLANK | 0.0360 | 0.0507 | 1.0000 | 20.000 | 0.0147 | 96.0109 | 340.4641 |
| | PA_BLANK_NFTrun8 | 14.04.2018_BLANK | 0.0340 | 0.0553 | 1.0000 | 20.000 | 0.0213 | 139.6522 | 495.2205 |
| | PA_BLANK_NFTrun9 | 14.04.2018_BLANK | 0.0350 | 0.0443 | 1.0000 | 20.000 | 0.0093 | 61.0978 | 216.6590 |
| | | | | | | Average | 0.0182 | 119.0438 | 422.1411 |
| | | | | | | SD | 0.0049 | 32.3121 | 114.5819 |
| | | | | | | LOD (SD*3) | 0.0148 | 96.9363 | 343.7457 |
| | | | | | | HALF LOD | 0.0074 | 48.4681 | 171.8728 |

| Set | Run | Sample identifier | Free Phosphorus | Total Phosphorus | Sample weight (g) | Extraction volume (mL) | delta-Abs (Phosphorus) | Total P* (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| Two | PA_BLANK_NFTrun1 | blankRun1 | 0.0423 | 0.0580 | 1.0000 | 20.000 | 0.0157 | 101.4743 | 359.8380 |
| | PA_BLANK_NFTrun2 | blankRun2 | 0.0350 | 0.0553 | 1.0000 | 20.000 | 0.0203 | 131.7007 | 467.0238 |
| | PA_BLANK_NFTrun3 | blankRun3 | 0.0340 | 0.0567 | 1.0000 | 20.000 | 0.0227 | 146.8139 | 520.6167 |
| | PA_BLANK_NFTrun4 | blankRun4 | 0.0373 | 0.0537 | 1.0000 | 20.000 | 0.0163 | 105.7924 | 375.1502 |
| | PA_BLANK_NFTrun5 | blankRun5 | 0.0337 | 0.0543 | 1.0000 | 20.000 | 0.0207 | 133.8597 | 474.6799 |
| | PA_BLANK_NFTrun6 | blankRun6 | 0.0343 | 0.0463 | 1.0000 | 20.000 | 0.0120 | 77.7250 | 275.6206 |
| | | | | | | Average | 0.0179 | 116.2277 | 412.1549 |
| | | | | | | SD | 0.0040 | 25.6703 | 91.0296 |
| | | | | | | LOD (SD*3) | 0.0119 | 77.0110 | 273.0888 |
| | | | | | | HALF LOD | 0.0059 | 38.5055 | 136.5444 |

| | | Sample identifier | Free Phosphorus | Total Phosphorus | Sample weight (g) | Extraction volume (mL) | delta-Abs (Phosphorus) | Total P (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| **Three** | PA_BLANK 12_NFTrun1 | BLANK 12 | 0.0327 | 0.0597 | 1.0000 | 20.000 | 0.0270 | 175.4925 | 622.3140 |
| | PA_BLANK 13_NFTrun2 | BLANK 13 | 0.0327 | 0.0547 | 1.0000 | 20.000 | 0.0220 | 142.9939 | 507.0707 |
| | PA_BLANK 12_NFTrun3 | BLANK 12 | 0.0330 | 0.0610 | 1.0000 | 20.000 | 0.0280 | 181.9923 | 645.3627 |
| | PA_BLANK 14_NFTrun4 | BLANK 14 | 0.0370 | 0.0617 | 1.0000 | 20.000 | 0.0247 | 160.3265 | 568.5338 |
| | PA_BLANK 14_NFTrun5 | BLANK 14 | 0.0370 | 0.0567 | 1.0000 | 20.000 | 0.0197 | 127.8279 | 453.2904 |
| | | | | | | Average | 0.0243 | 157.7266 | 559.3143 |
| | | | | | | SD | 0.0035 | 22.4844 | 79.7320 |
| | | | | | | LOD (SD*3) | 0.0104 | 67.4532 | 239.1959 |
| | | | | | | HALF LOD | 0.0052 | 33.7266 | 119.5979 |

| Set | n | Mean | Minimum | Maximum | SD | LOQ |
|---|---|---|---|---|---|---|
| **Leaf samples** | | | | | | |
| One | 52 | 1524.617 | 171.873 | 5347.971 | 1221.784 | 7633.535 |
| Two | 48 | 1457.615 | 136.544 | 4865.073 | 821.265 | 5563.939 |
| Three | 48 | 2125.368 | 376.720 | 4731.758 | 1019.516 | 7222.947 |
| **Wholegrain flour samples** | | | | | | |
| One | 44 | 11996.104 | 4529.268 | 28178.463 | 4546.271 | 34727.461 |
| Two | 48 | 13084.577 | 5948.193 | 19831.653 | 2997.405 | 28071.601 |
| Three | 49 | 14607.098 | 10495.986 | 20911.322 | 2447.260 | 26843.397 |

*Total P, Phytic acid-P, the name was not changed on these tables because this is how it is referred to by the Megazyme kit's manufacturer; SD, standard deviation; LOD, Limit of Detection; LOQ, Limit of Quantification; NFT, Nutrient Film Technique.

## 9.2 APPENDIX 2

### 9.2.1 Blank data for limit of detection (LOD).

Table 9-15 Blank raw data.

| Harvest year | Sample identifier | Free Phosphorus (Abs650 nm) | Total Phosphorus (Abs650 nm) | Sample weight (g) | Extraction volume (mL) | ΔAbs (Phosphorus) | Total Phosphorus (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 1 | PA_BLANK 1_run1 | 0.0350 | 0.0603 | 1.00 | 20.00 | 0.0253 | 160.4978 | 569.1413 |
| | PA_BLANK 2_run2 | 0.0353 | 0.0557 | 1.00 | 20.00 | 0.0203 | 128.8206 | 456.8108 |
| | PA_BLANK 3_run3 | 0.0357 | 0.0557 | 1.00 | 20.00 | 0.0200 | 126.7088 | 449.3221 |
| | PA_BLANK 4_run4 | 0.0367 | 0.0613 | 1.00 | 20.00 | 0.0247 | 156.2742 | 554.1639 |
| | PA_BLANK 5_run5 | 0.0347 | 0.0560 | 1.00 | 20.00 | 0.0213 | 135.1561 | 479.2769 |
| | PA_BLANK 6_run6 | 0.0340 | 0.0527 | 1.00 | 20.00 | 0.0187 | 118.2616 | 419.3673 |
| | PA_BLANK 7_run7 | 0.0333 | 0.0567 | 1.00 | 20.00 | 0.0233 | 147.8270 | 524.2091 |
| | PA_BLANK 10_run10 | 0.0380 | 0.0613 | 1.00 | 20.00 | 0.0233 | 147.8270 | 524.2091 |
| | PA_BLANK 11_run11 | 0.0347 | 0.0623 | 1.00 | 20.00 | 0.0277 | 175.2805 | 621.5622 |
| | PA_BLANK 12_run12 | 0.0387 | 0.0483 | 1.00 | 20.00 | 0.0097 | 61.2426 | 217.1723 |
| | PA_BLANK 13_run13 | 0.0350 | 0.0610 | 1.00 | 20.00 | 0.0260 | 164.7215 | 584.1187 |
| | PA_BLANK 14_run14 | 0.0347 | 0.0567 | 1.00 | 20.00 | 0.0220 | 139.3797 | 494.2543 |
| 2 | PA_BLANK 1_run15 | 0.0360 | 0.0513 | 1.00 | 20.00 | 0.0153 | 98.9951 | 351.0465 |
| | PA_BLANK 2_run16 | 0.0397 | 0.0623 | 1.00 | 20.00 | 0.0227 | 146.3406 | 518.9383 |
| | PA_BLANK 3_run17 | 0.0377 | 0.0593 | 1.00 | 20.00 | 0.0217 | 139.8844 | 496.0440 |
| | PA_BLANK 4_run18 | 0.0353 | 0.0603 | 1.00 | 20.00 | 0.0250 | 161.4051 | 572.3584 |
| | PA_BLANK 5_run19 | 0.0380 | 0.0573 | 1.00 | 20.00 | 0.0193 | 124.8199 | 442.6239 |
| | PA_BLANK 6_run20 | 0.0353 | 0.0590 | 1.00 | 20.00 | 0.0237 | 152.7968 | 541.8327 |
| | PA_BLANK 4 REP_run20 | 0.0357 | 0.0660 | 1.00 | 20.00 | 0.0303 | 195.8382 | 694.4616 |
| | PA_BLANK 4 REP_run20 | 0.0340 | 0.0613 | 1.00 | 20.00 | 0.0273 | 176.4696 | 625.7786 |
| | PA_BLANK 7_run21 | 0.0340 | 0.0650 | 1.00 | 20.00 | 0.0310 | 200.1423 | 709.7245 |
| | PA_BLANK 8_run22 | 0.0350 | 0.0577 | 1.00 | 20.00 | 0.0227 | 146.3406 | 518.9383 |
| | PA_BLANK 9_run23 | 0.0340 | 0.0583 | 1.00 | 20.00 | 0.0243 | 157.1009 | 557.0955 |

| Harvest year | Sample identifier | Free Phosphorus (Abs650 nm) | Total Phosphorus (Abs650 nm) | Sample weight (g) | Extraction volume (mL) | ΔAbs (Phosphorus) | Total Phosphorus (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| | PA_BLANK 10_run24 | 0.0340 | 0.0627 | 1.00 | 20.00 | 0.0287 | 185.0778 | 656.3043 |
| | PA_BLANK 10_run25 | 0.0330 | 0.0583 | 1.00 | 20.00 | 0.0253 | 163.5571 | 579.9899 |
| | PA_BLANK 10_run26 | 0.0330 | 0.0510 | 1.00 | 20.00 | 0.0180 | 116.2117 | 412.0981 |
| | PA_BLANK 11_run27 | 0.0357 | 0.0487 | 1.00 | 20.00 | 0.0130 | 83.9306 | 297.6264 |

### 9.2.2 White flour and wholegrain flour summary statistics

Table 9-16 Phytic acid summary statistics.

| Sample type | Harvest year | n | NMV | Mean | Median | Minimum | Maximum | SD | SE | Var |
|---|---|---|---|---|---|---|---|---|---|---|
| White flour | 1 | 129 | 0 | 1430.1 | 1390.2 | 157.4 | 3071.3 | 553.75 | 48.8 | 306640.9 |
| White flour | 2 | 113 | 1 | 414.7 | 374.5 | 178.6 | 1563.6 | 289.13 | 27.2 | 83593.6 |
| Wholegrain flour | 1 | 129 | 0 | 9594.5 | 9650.8 | 4853.3 | 13219.5 | 1794.96 | 158.0 | 3221879.7 |
| Wholegrain flour | 2 | 113 | 1 | 9122.3 | 9100.7 | 5557.6 | 12332.9 | 1344.94 | 126.5 | 1808860.9 |

Data are in mg $kg^{-1}$, n, number of values; NMV, number of missing values; SD, standard deviation; SE, standard error; Var, variance.

### 9.2.3 Oat flour reference material

Table 9-17 Oat flour reference material phytic acid raw data.

| Harvest year | Sample identifier | Free Phosphorus (Abs650 nm) | Total Phosphorus (Abs650 nm) | Sample weight (g) | Extraction volume (mL) | ΔAbs (Phosphorus) | Total Phosphorus (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 1 | OAT POWDER_run1 | 0.1293 | 0.5563 | 1.0079 | 20.00 | 0.4270 | 2712.0665 | 9617.2571 |
| | OAT POWDER_run2 | 0.1437 | 0.7050 | 1.0079 | 20.00 | 0.5613 | 3567.0105 | 12648.9733 |
| | OAT POWDER_run3 | 0.1300 | 0.6740 | 1.0079 | 20.00 | 0.5440 | 3488.0459 | 12368.9571 |
| | OAT POWDER_run4 | 0.1490 | 0.7267 | 1.0079 | 20.00 | 0.5777 | 3705.1335 | 13138.7712 |
| | OAT POWDER_run5 | 0.1467 | 0.6957 | 1.0673 | 20.00 | 0.5490 | 3349.2652 | 11876.8268 |
| | OAT POWDER_run6 | 0.1663 | 0.7470 | 1.0673 | 20.00 | 0.5807 | 3356.1187 | 11901.1301 |
| | OAT POWDER_run7 | 0.1427 | 0.6193 | 1.0049 | 20.00 | 0.4767 | 3042.1756 | 10787.8568 |
| | OAT POWDER_run7 | 0.1430 | 0.7043 | 1.0215 | 20.00 | 0.5613 | 3524.3157 | 12497.5734 |
| | OAT POWDER_run8 | 0.1480 | 0.7153 | 1.0076 | 20.00 | 0.5673 | 3321.7148 | 11779.1304 |
| | OAT POWDER_run9 | 0.1917 | 0.8087 | 1.0076 | 20.00 | 0.6170 | 4041.1925 | 14330.4699 |
| | OAT POWDER_run9 | 0.0750 | 0.4307 | 1.0042 | 20.00 | 0.3557 | 2337.4131 | 8288.6991 |
| | OAT POWDER_run9 | 0.0627 | 0.3997 | 1.0177 | 20.00 | 0.3370 | 2185.3583 | 7749.4977 |
| | OAT POWDER_run9 | 0.0543 | 0.3783 | 1.0169 | 20.00 | 0.3240 | 2102.7096 | 7456.4170 |
| | OAT POWDER_run10 | 0.1200 | 0.4690 | 1.0314 | 20.00 | 0.3490 | 2082.7641 | 7385.6883 |
| | OAT POWDER_run11 | 0.1483 | 0.5690 | 1.0314 | 20.00 | 0.4207 | 2500.3961 | 8866.6528 |
| | OAT POWDER_run12 | 0.1497 | 0.6247 | 1.0314 | 20.00 | 0.4750 | 2948.2913 | 10454.9338 |
| | OAT POWDER_run13 | 0.1753 | 0.5890 | 1.0314 | 20.00 | 0.4137 | 2586.6709 | 9172.5917 |
| 2 | OAT POWDER_run15 | 0.1163 | 0.4817 | 1.0184 | 20.00 | 0.3653 | 2285.4978 | 8104.6023 |
| | OAT POWDER_run16 | 0.1417 | 0.5230 | 1.0236 | 20.00 | 0.3813 | 2392.7677 | 8484.9920 |
| | OAT POWDER_run17 | 0.1427 | 0.5320 | 1.0163 | 20.00 | 0.3893 | 2454.8654 | 8705.1963 |
| | OAT POWDER_run18 | 0.1430 | 0.4470 | 1.0294 | 20.00 | 0.3040 | 1883.4843 | 6679.0223 |
| | OAT POWDER_run19 | 0.1413 | 0.4510 | 1.0243 | 20.00 | 0.3097 | 1922.8230 | 6818.5211 |
| | OAT POWDER_run20 | 0.1420 | 0.4807 | 1.0032 | 20.00 | 0.3387 | 2162.1483 | 7667.1927 |
| | OAT POWDER_run20 | 0.1432 | 0.3843 | 1.0294 | 20.00 | 0.2412 | 1500.4920 | 5320.8935 |

| Harvest year | Sample identifier | Free Phosphorus (Abs650 nm) | Total Phosphorus (Abs650 nm) | Sample weight (g) | Extraction volume (mL) | ΔAbs (Phosphorus) | Total Phosphorus (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| | OAT POWDER_run21 | 0.1507 | 0.6497 | 1.0033 | 20.00 | 0.4990 | 3222.7379 | 11428.1484 |
| | OAT POWDER_run22 | 0.1387 | 0.3377 | 1.0021 | 20.00 | 0.1990 | 1284.9375 | 4556.5159 |
| | OAT POWDER_run23 | 0.1420 | 0.3993 | 1.0080 | 20.00 | 0.2573 | 1632.5321 | 5789.1211 |
| | OAT POWDER_run24 | 0.1373 | 0.4467 | 1.0041 | 20.00 | 0.3093 | 2027.2140 | 7188.7020 |
| | OAT POWDER_run25 | 0.1413 | 0.4553 | 1.0041 | 20.00 | 0.3140 | 2066.5723 | 7328.2707 |
| | OAT POWDER_run26 | 0.1393 | 0.5277 | 1.0041 | 20.00 | 0.3883 | 2545.4339 | 9026.3612 |
| | OAT POWDER_run27 | 0.1400 | 0.5170 | 1.0079 | 20.00 | 0.3770 | 2435.1589 | 8635.3152 |

Abs, Absorbance.

### 9.2.4 Laboratory Reference Material (LRM)

Table 9-18 Laboratory Reference Material raw data.

| Sample identifier | Free Phosphorus (Abs650 nm) | Total Phosphorus (Abs650 nm) | Sample weight (g) | Extraction volume (mL) | ΔAbs (Phosphorus) | Total Phosphorus (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|
| LRM 1_run15 | 0.0577 | 0.4280 | 1.0323 | 20.00 | 0.3703 | 2285.5819 | 8104.9003 |
| LRM 2_run16 | 0.0670 | 0.3860 | 1.0239 | 20.00 | 0.3190 | 2001.0558 | 7095.9425 |
| LRM 3_run17 | 0.0717 | 0.3987 | 1.0344 | 20.00 | 0.3270 | 2025.7566 | 7183.5339 |
| LRM 4_run18 | 0.0690 | 0.3913 | 1.0200 | 20.00 | 0.3223 | 2015.4760 | 7147.0780 |
| LRM 5_run19 | 0.0697 | 0.3877 | 1.0157 | 20.00 | 0.3180 | 1991.2862 | 7061.2985 |
| LRM 6_run20 | 0.0740 | 0.3873 | 1.0236 | 20.00 | 0.3133 | 1960.5453 | 6952.2883 |
| LRM 4 REP_run20 | 0.0675 | 0.3523 | 1.0200 | 20.00 | 0.2848 | 1788.5093 | 6342.2315 |
| LRM 7_run21 | 0.0723 | 0.3590 | 1.0235 | 20.00 | 0.2867 | 1814.8661 | 6435.6955 |
| LRM 7_run22 | 0.0710 | 0.3783 | 1.0235 | 20.00 | 0.3073 | 1942.9508 | 6889.8965 |
| LRM 9_run23 | 0.0690 | 0.3547 | 0.9946 | 20.00 | 0.2857 | 1836.6963 | 6513.1073 |
| LRM 10_run24 | 0.0693 | 0.3833 | 1.0127 | 20.00 | 0.3140 | 2040.3218 | 7235.1837 |
| LRM 10_run25 | 0.0733 | 0.3930 | 1.0127 | 20.00 | 0.3197 | 2086.0008 | 7397.1660 |
| LRM 10_run26 | 0.0723 | 0.3977 | 1.0127 | 20.00 | 0.3253 | 2114.3743 | 7497.7811 |
| LRM 11_run27 | 0.0713 | 0.4303 | 1.0067 | 20.00 | 0.3590 | 2321.6555 | 8232.8209 |

Abs, Absorbance.

## 9.3 APPENDIX 3

### 9.3.1 Grain phosphorus, zinc and grain yield of Indian wheat genotypes selected for phytic acid analysis.

Table 9-19 Indian wheat grain P, Zn and GYD. Data for elements was obtained by ICP-MS.

| Genotype | Site | Element/GYD | Obs | Mean | Variance | SD | SE |
|---|---|---|---|---|---|---|---|
| BH_1146 | Hisar | Phosphorus | 4 | 3559 | 155566 | NA | 197.2 |
| | Hisar | Zinc | 4 | 36.03 | 65.35 | NA | 4.042 |
| | Hisar | GYD | 4 | 2.99 | 0.4361 | 0.6604 | 0.3302 |
| | Karnal | Phosphorus | 4 | 3850 | 43806 | NA | 104.6 |
| | Karnal | Zinc | 4 | 35.84 | 28.86 | NA | 2.686 |
| | Karnal | GYD | 4 | 4.329 | 0.5351 | 0.7315 | 0.3658 |
| | Kumarganj-sodic | Phosphorus | 3 | 3813 | 185434 | NA | 248.6 |
| | Kumarganj-sodic | Zinc | 3 | 29.54 | 23.86 | NA | 2.82 |
| | Kumarganj-sodic | GYD | 4 | 1.55 | 0.9356 | 0.9673 | 0.4836 |
| KHARCHIA_65 | Hisar | Phosphorus | 4 | 3800 | 270018 | NA | 259.8 |
| | Hisar | Zinc | 4 | 40.16 | 121.03 | NA | 5.501 |
| | Hisar | GYD | 4 | 1.639 | 0.1438 | 0.3792 | 0.1896 |
| | Karnal | Phosphorus | 4 | 3877 | 61577 | NA | 124.1 |
| | Karnal | Zinc | 4 | 38.21 | 64.22 | NA | 4.007 |
| | Karnal | GYD | 4 | 1.752 | 1.3568 | 1.1648 | 0.5824 |
| | Kumarganj-sodic | Phosphorus | 3 | 3870 | 245198 | NA | 285.9 |
| | Kumarganj-sodic | Zinc | 3 | 34.94 | 15 | NA | 2.236 |
| | Kumarganj-sodic | GYD | 4 | 1.1 | 0.0051 | 0.0712 | 0.0356 |
| KRL_3-4 | Hisar | Phosphorus | 4 | 3034 | 423084 | NA | 325.2 |
| | Hisar | Zinc | 4 | 27.15 | 27.78 | NA | 2.635 |
| | Hisar | GYD | 4 | 2.898 | 0.3184 | 0.5643 | 0.2821 |
| | Karnal | Phosphorus | 4 | 4056 | 14385 | NA | 60 |
| | Karnal | Zinc | 4 | 40.86 | 11.06 | NA | 1.663 |
| | Karnal | GYD | 4 | 1.512 | 0.8277 | 0.9098 | 0.4549 |
| | Kumarganj-sodic | Phosphorus | 3 | 3790 | 166770 | NA | 235.8 |
| | Kumarganj-sodic | Zinc | 3 | 28.26 | 10.81 | NA | 1.898 |
| | Kumarganj-sodic | GYD | 4 | 1.065 | 0.1796 | 0.4238 | 0.2119 |
| HD_2932 | Hisar | Phosphorus | 4 | 2924 | 498276 | NA | 352.9 |
| | Hisar | Zinc | 4 | 25.64 | 36.11 | NA | 3.005 |
| | Hisar | GYD | 4 | 3.549 | 0.1008 | 0.3176 | 0.1588 |

| Genotype | Site | Element/GYD | Obs | Mean | Variance | SD | SE |
|---|---|---|---|---|---|---|---|
| | Karnal | Phosphorus | 4 | 2829 | 619951 | NA | 393.7 |
| | Karnal | Zinc | 4 | 23.87 | 58.19 | NA | 3.814 |
| | Karnal | GYD | 4 | 5.33 | 0.5381 | 0.7335 | 0.3668 |
| | Kumarganj-sodic | Phosphorus | 3 | 3434 | 51557 | NA | 131.1 |
| | Kumarganj-sodic | Zinc | 3 | 24.44 | 4.14 | NA | 1.175 |
| | Kumarganj-sodic | GYD | 4 | 1.65 | 0.35 | 0.5916 | 0.2958 |
| HW_2044 | Hisar | Phosphorus | 4 | 2900 | 360878 | NA | 300.4 |
| | Hisar | Zinc | 4 | 24.51 | 111.92 | NA | 5.29 |
| | Hisar | GYD | 4 | 3.032 | 2.4838 | 1.576 | 0.788 |
| | Karnal | Phosphorus | 4 | 3471 | 497331 | NA | 352.6 |
| | Karnal | Zinc | 4 | 29.24 | 117.33 | NA | 5.416 |
| | Karnal | GYD | 4 | 5.712 | 0.1077 | 0.3282 | 0.1641 |
| | Kumarganj-sodic | Phosphorus | 3 | 3354 | 619159 | NA | 454.3 |
| | Kumarganj-sodic | Zinc | 3 | 23.49 | 35.98 | NA | 3.463 |
| | Kumarganj-sodic | GYD | 4 | 1.332 | 0.047 | 0.2169 | 0.1084 |
| WH_1021 | Hisar | Phosphorus | 4 | 2989 | 3204649 | NA | 895.1 |
| | Hisar | Zinc | 4 | 29.02 | 394.94 | NA | 9.937 |
| | Hisar | GYD | 4 | 3.985 | 0.5502 | 0.7417 | 0.3709 |
| | Karnal | Phosphorus | 4 | 3318 | 130803 | NA | 180.8 |
| | Karnal | Zinc | 4 | 28.66 | 34.21 | NA | 2.924 |
| | Karnal | GYD | 4 | 6.187 | 0.4033 | 0.6351 | 0.3175 |
| | Kumarganj-sodic | Phosphorus | 3 | 3586 | 382832 | NA | 357.2 |
| | Kumarganj-sodic | Zinc | 3 | 26.19 | 16.8 | NA | 2.366 |
| | Kumarganj-sodic | GYD | 4 | 1.165 | 0.3329 | 0.577 | 0.2885 |

Data for elements was obtained by ICP-MS. Element concentrations are expressed in mg $kg^{-1}$, GYD is expressed in t $ha^{-1}$. GYD, grain yield; Obs, number of observations; SD, standard deviation; SE, standard error of the mean.

### 9.3.2 Blank data for Limit of Detection (LOD)

Table 9-20 Blank raw data.

| | Free Phosphorus | Total Phosphorus | Sample weight (g) | Extraction volume (mL) | ΔAbs (Phosphorus) | Total Phosphorus (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|
| PA_BLANK 12_IndianRun0 | 0.0340 | 0.0533 | 1.0000 | 20.000 | 0.0193 | 108.3303 | 384.1500 |
| PA_BLANK 12_IndianRun1 | 0.0340 | 0.0543 | 1.0000 | 20.000 | 0.0203 | 113.9336 | 404.0198 |
| PA_BLANK 12_IndianRun2 | 0.0347 | 0.0520 | 1.0000 | 20.000 | 0.0173 | 97.1237 | 344.4103 |
| PA_BLANK 12_IndianRun3 | 0.0340 | 0.0523 | 1.0000 | 20.000 | 0.0183 | 102.7270 | 364.2802 |
| | | | | Average | 0.0188 | 105.5287 | 374.2151 |
| | | | | SD | 0.0013 | 7.2338 | 25.6518 |
| | | | | LOD | 0.0039 | 21.7015 | 76.9555 |

ΔAbs, Difference in Absorbance; SD, standard deviation; LOD, Limit of Detection.

### 9.3.3 Oat flour reference material

Table 9-21 Oat flour reference material phytic acid data (n=4).

| | Free Phosphorus | Total Phosphorus | Sample weight (g) | Extraction volume (mL) | ΔAbs (Phosphorus) | Total Phosphorus (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|
| PA_C+ OATS_IndianRun0 | 0.1480 | 0.5867 | 1.0027 | 20.000 | 0.4387 | 2378.1844 | 8433.2782 |
| PA_C+ OATS 12_IndianRun1 | 0.0600 | 0.4953 | 1.0284 | 20.000 | 0.4353 | 2733.0658 | 9691.7225 |
| PA_C+ OATS 12_IndianRun2 | 0.0857 | 0.5093 | 1.0284 | 20.000 | 0.4237 | 2631.5501 | 9331.7381 |
| PA_C+ OATS 12_IndianRun3 | 0.0630 | 0.5333 | 1.0284 | 20.000 | 0.4703 | 2957.9934 | 10489.3384 |
| | | | | Average | 0.4420 | 2675.1984 | 9486.5193 |
| | | | | SD | 0.0200 | 240.4469 | 852.6486 |

ΔAbs, Difference in Absorbance; SD, standard deviation.

### 9.3.4 Laboratory reference material (lrm)

Table 9-22 Laboratory reference material (LRM) data, n=3.

| | Free Phosphorus | Total Phosphorus | Sample weight (g) | Extraction volume (mL) | ΔAbs (Phosphorus) | Total Phosphorus (mg $kg^{-1}$) | Phytic acid (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|
| PA_LRM 12_IndianRun1 | 0.0713 | 0.4010 | 1.0122 | 20.000 | 0.3297 | 2102.8047 | 7456.7541 |
| PA_LRM 12_IndianRun2 | 0.0700 | 0.3980 | 1.0122 | 20.000 | 0.3280 | 2069.9361 | 7340.1988 |
| PA_LRM 12_IndianRun3 | 0.0710 | 0.4120 | 1.0122 | 20.000 | 0.3410 | 2178.9214 | 7726.6716 |
| | | | | Average | 0.3329 | 2117.2207 | 7507.8748 |
| | | | | SD | 0.0071 | 55.9045 | 198.2430 |

ΔAbs, Difference in Absorbance; SD, standard deviation.

### 9.3.5 Indian wheat wholegrain flour summary statistics

Table 9-23 Summary statistics of Indian wheat phytic acid concentration mg $kg^{-1}$.

| n | Mean | Median | Min | Max | Q1 | Q3 | SD | SE | Var | LOQ |
|---|---|---|---|---|---|---|---|---|---|---|
| 72 | 8371.86 | 8515.09 | 4306.79 | 11962.87 | 6994.54 | 9652.96 | 1978.49 | 233.17 | 3914416.52 | 18264.31 |

n, number of samples; min, minimum value; max, maximum value; Q1, first quartile; Q3, third quartile; SD, standard deviation; SE, standard error; Var, variance; LOQ, Limit of Quantification.