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Development and evaluation of low-phytate wheat germplasm to reduce diffuse phosphate pollution from pig and poultry production units

by

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1. ABSTRACT

The aim of this project was to reduce phosphate pollution from monogastric farm animals by changing the availability of phosphate from wheat. Currently a lot of wheat derived phosphate is stored in a non-available form, as phytate. The approaches taken in this research project were to: i) determine the effect of high available phosphate (HAP) wheat on phosphorous excretion in pigs and poultry ii) develop a model for quantifying the effect of HAP wheat on diffuse pollution reduction iii) develop adapted wheat germplasm, iv) development of a toolkit for marker-assisted breeding of the HAP trait and v) determine the effect of P fertiliser treatment on the growth and phosphate metabolism of HAP wheat.

Five feeding trials were undertaken using both pigs and poultry to demonstrate the potential reduction in phosphate excretion when HAP wheat was used in formulated diets. The animal performance was not as anticipated, with poultry showing an apparent increase in the availability of P when conventional wheat was fed but with a reduced total P content in the diet (as a result of reducing the amount of inorganic P added to the diet). However, the results were confounded by differences in the protein contents of the HAP and conventional wheat. Specialised diets were therefore produced to start understanding the potential mechanisms involved. The results demonstrated that under certain conditions, P excretion could be reduced in both pigs and poultry using HAP wheat. This can also be achieved (in poultry) using conventional wheat, and reducing the amount of dicalcium phosphate added to the diet. However, when this was done, the growth rate of the birds was reduced.

Using the data from our feeding trials it can be calculated that by replacing conventional feed with a low phytate alternative it would be possible to reduce P load to GB waters by 0.53% (321 tonnes P per year) and the agricultural contribution to the total P load to GB waters by 2.73%. As the low availability of phytate P is a feature of digestion in all monogastric animals, it could be envisaged that the development of HAP wheat would also have an impact on P excretion in both the industrial fish farming and human nutrition sectors.

Adapted wheat germplasm was developed using three different techniques. This has provided germplasm with several different mutations resulting in the reduction of phytate and potentially an increased uptake of phosphate. Within the period of the project, full characterisation has not been possible but adapted material has been developed. The development of the toolkit for marker-assisted breeding will improve the efficiency of this process.

There was no evidence for altered performance or need for altered agronomy with HAP wheat. This will need to be confirmed with the UK adapted material and the new mutations. However,

since phytate is associated with Fe, Zn etc., the micronutrient levels should be monitored when new germplasm is developed as changes were seen in the analysis conducted on the US variety.

Although significant advantages were shown with the use of low-phytate wheat (LPW) in this project the value of such a commercial development has been superseded by other technologies. The production cost and efficacy of phytase enzymes has improved, such the cost of adding these to formulated feeds for monogastrics is very cheap. In addition, the pig breeding industry has developed GM pigs modified to produce phytase in their saliva. The plant breeding industry therefore considers that the development of a commercial LPW variety would provide no competitive advantage and that the development of other traits would be a better use of their efforts.

However the lines developed in this program are still of interest in some third world countries where human diets are generally poor and changing the availability of micronutrients has significant health benefits.

2. SUMMARY

The purpose of this project was to reduce diffuse phosphate pollution through the production of new improved wheat lines. The consortium developed UK-adapted wheat germplasm with a lower phytate content in the grain, thus increasing bioavailability of phosphate to monogastric animals. This in turn reduced the level of phosphates entering the environment from animal wastes. The precise effect of changes in feed quality was determined through chicken and pig feeding studies using University of Idaho material. The consortium has started mapping the genes involved in this pathway to understand the plant physiology underlying changes in phytate content, together with related mineral nutrition. In addition to the original source of low phytate germplasm, a structured mutation population was evaluated to identify potential new candidates for low phytate lines. Finally, the net effect on the environment, industry and diffuse pollution has been modelled in this project.

The major form in which phosphorus (P) occurs in plants is myo-inositol-1,2,3,4,5,6-hexakisphosphate, commonly referred to as phytic acid, or InsP6; it is an important anti-nutritional factor to farm animals due to its ability to complex micro-nutrients such as iron and zinc. It occurs in different tissues but accumulates in high amounts in the seeds where it serves as a storage form of *myo*-inositol and phosphorus for utilisation during seed germination and seedling growth. Phytic acid biosynthesis in developing seeds is not very well understood although much progress has been made in understanding the biosynthesis pathway from the analysis of mutant lines exhibiting a low phytic acid (*lpa)* phenotype. Studies on maize, barley, rice and soybean in particular have shown that genetic lesions in several distinct gene classes can lead to lower levels of seed phytate.

Production of microbial-derived phytase, which can be added to the diets of pigs and poultry, is undertaken on a commercial scale as part of a feed enzyme market that is now extremely large. Exogenous phytase is an effective means of breaking down a proportion of the phytate in plant-derived feeds and thereby rendering the P, as well as other minerals and certain proteins, available to the animal. By using a careful balance of nutrients combined with phytase (together with other measures), the livestock industry in the Netherlands has halved the amount of P excreted by growing and finishing pigs over the last 20 years (Lenis and Jongbloed, 1999). However, these beneficial effects of microbial phytase are adversely affected by a high ratio of calcium: total phosphorus (Brady *et al*., 2002). Since the calcium content of layer hen diets is high (and the Ca:P ratio is also high), microbial phytases would not be efficacious and so are not normally added to their ration.

Total pig output in the UK in terms of number of pigs is 9.5m/annum (down from 14+ m in 1998) and they consume 2.7 m tonnes of feed comprising 50–55% wheat. It is estimated that 9,720

tonnes of P_2O_2 is excreted by pigs each year in the UK. The impact that low P availability has on the total P contents in pig diets is illustrated in Table 1. Pig manure is generally utilised as efficiently as possible, however, where pig manure is spread, soil content of P has increase over a number of years. This is exacerbated by the 150,000 outdoor sows where waste output diffuses across land that is un-cropped for up to two years.

Table 1. The maximum potential loss of P from pigs to the environment in the UK.

	Kg Feed per pig produced (MLC 2004 Pig Year book)	Digestible P required g/kg (Whittemore *et al.,* 2003)	Total P supplied in typical diets g/kg.	Max. potential loss to the environment kg/pig
Sow feed	66.5	2.75	8	0.35
Weaner feed	48.5	3.4	9	0.27
Finisher feed	168.0	2.4	8	0.94
Total	283.0	NA		1.56

Following the removal of bone meal as a traditional source of P, there is now a greater reliance on adding phosphates in the form of dicalcium phosphate or the less digestible rock phosphates. An increasing number of supplement manufacturers include phytase in pig diets, which results in a combination of reduced total P in the diet and increased P availability (up to 60% available across a number of studies; Jongbloed *et al.,* 2004; Pallauf *et al.,* 1993; Hoppe *et al.,* 1993). Inclusion of phytases have been shown to reduce faecal output of P by up to 30% (down from 625 g P/pig finished to 428 g P/pig finished). These data indicate that, while exogenous phytases can significantly reduce P excretion by pigs, there is scope, by the use of high available phosphate (HAP) wheat in the diet, to further reduce diffuse P pollution from pig enterprises.

2.1. Programme of work

The work in this proposal was divided into 5 work packages; only after preliminary work in WP1 had confirmed that HAP wheat demonstrates the same value as seen for HAP maize and barley, was considerable effort in WP2-5 justified. These latter work packages developed commercial germplasm (WP3), markers for the breeding industry (WP4); determined the effects of P fertiliser treatment on the growth and phosphate metabolism of HAP wheat (WP5). Data from these investigations was used to develop a model (WP2) to predict and quantify phosphorous budgets in terms of metabolism in the animal and subsequent losses into the environment. The output from WP1&2 will be important in informing policy regarding measures to reduce pollution that can be used to offset livestock number reductions which may otherwise be required to effect reduced pollution reduction.

2.1.1. Work Package 1. Effect of HAP wheat on phosphorus excretion in pigs and poultry

Samples of HAP wheat were compared with conventional feed wheat in the presence or absence of exogenous phytase. Diets containing these wheats (and phytases) were fed to groups of monogastric livestock. In the first instance, broilers were used and this study was informed the planning for other trials. Feed intakes, growth rates, feed conversion efficiencies and phosphorus balances were determined in each case. The effect of wheat type, added phytase and interactions between wheat type and added phytase were considered on each of these parameters. This was then used to define and initiate quantification of the potential value of HAP wheat for the industry in terms of the reduction in P excretion that it brings about. The University of Reading and Harper Adams University College led this work with technical assistance from Frank Wright, ABN and MLC.

2.1.2. Work Package 2. Develop a model for quantifying effect of HAP wheat on diffuse P pollution reduction.

The data from Work Package 1, was used to develop a model to predict phosphorus losses by pigs and poultry in the UK and estimate the contribution that HAP wheat may make (compared with exogenous phytase) in ameliorating these losses. This model took into account the relative cost of different amelioration strategies to enable the development of a decision support system that could be used for selecting appropriate strategies in different situations and be important in informing policy. The University of Nottingham and the Scottish Crop Research Institute led this work, with inputs from Harper Adams University College, The University of Reading and NIAB. The industry partners had a key input into this work package, including Anglian Water and the Environment agency.

2.1.3. Work Package 3. Development of Germplasm; Three types of material were developed in this work package for three distinct purposes.

1. **Trait introgression:** In close co-operation with Limagrain UK Ltd (formally Advanta Seeds), a crossing program was undertaken to transfer the low phytate trait from the University of Idaho low phytate wheat line Js-12-Mu-6 into Northern European adapted material. Nine of the most competitive new lines from the Limagrain crossing programs and a small selection of established feed wheat varieties were used as parental material. This was backcrossed for three generations and the progeny assayed from the F_2 generation onwards to confirm the presence of the low phytate trait. By backcrossing with these elite lines we hoped to speed up the development of genetically uniform commercially competitive elite lines containing the low phytate trait.

2. **Development of a DH mapping population for the Js-12-Mu-6 HAP wheat**: one of the Js-12-Mu-6 x UK elite crosses described above was chosen for generation of a large (min. 200 lines) doubled haploid mapping population to facilitate the objectives of WP4.
3. **Development of a mutant population**: in order to prospect for new sources of reduced phytic acid, Paragon M6 seed stocks, developed through the Wheat Genetic Improvement Network (WGIN) and held at John Innes Centre (JIC), were assayed using the wheat flour colorimetric test. Candidates identified using the colorimetric assay have been partially characterised biochemically to determine possible effects on pathway intermediates and complexed ions.

2.1.4. Work Package 4. Development of a toolkit for marker-assisted breeding of the HAP trait.

The objectives of this WP were:

1. **To fine map the genes responsible for the low phytic acid phenotype of the Js-12-Mu-6 donor line** utilised in this programme. This activity was guided by pre-existing knowledge supplied by the University of Idaho, and screening for marker polymorphisms, facilitated by large marker sets available through ongoing wheat diversity and mapping work in NIAB and Limagrain.
2. **To characterise in detail the myo-inositol 3-phosphate synthase (MIPS) family of genes from the hexaploid wheat genome.** This provided markers to rule in or out candidates affected in the Js-12-Mu-6 and other mutant lines generated de novo in the programme.
3. **To isolate a panel of new HAP mutants from the EMS mutant population using phenotypic screens.** This work utilised a new mutant population in a high-yielding spring feed wheat background (Cadenza) for isolation of mutants with high HAP levels, which may offer different advantages and disadvantages for breeders as well as being able to test the hypothesis that particular MIPS genes are critical in conditioning overall phytate levels in the wheat grain. The mutant lines can then be characterised for MIPS gene expression levels, subjected to quantitative HPLC analysis of levels of InsP6 as well as the range of phosphorylated intermediates.
4. **To relate all new mutations identified to candidate genes and to each other**. This work involved sequencing candidate genes from a series of mutant lines to determine if there are suspect lesions that might explain those phenotypes.

Ultimately, the overall aim via the above tasks was to generate novel molecularly tagged phenotypic variation to underpin more effective breeding for HAP wheat. This work evolved significantly during the program due to the rapid changes in technology and new resources becoming available to the project.

2.1.5. Work Package 5. Determine the effects of P fertiliser treatment on the growth and phosphate metabolism of HAP wheat

Existing specialist field sites were used to study the growth and phosphate metabolism of the natural (spring-sown) low phytate wheat germplasm. The original spring-sown variety, from which the low-phytate germplasm was developed, was used as a control. Three replicate P-response gradients (P Indices between 3 and 9; MAFF, 2000) were established using broadcast triple superphosphate (TSP) in the low P-fertility Wharf Ground Field at Wellesbourne, the University of Warwick (Greenwood *et al*., 2005).

Overall Conclusions

The feeding of LPW in combination with phytase enzymes had a significant additive effect in pigs. The increased availability of the plant derived phosphate, reduction the total excreted phosphate and the need for adding some of the phosphate to the diet. A model was developed to show the possible environmental benefits of this work.

New wheat germplasm was developed for this project and has been made available to the plant breeding industry. The detailed work necessary for the development of marker based selection requires further investment to complete the work. This is still technically quite difficult and expensive as the wet chemistry necessary for this work is still not completely reliable.

However, since this project started there have been other technical developments within the feed industry which have started to provide more cost effective solutions. These include the development of pigs with phytase enzymes in their saliva and the feed industry has taken advantage of cheaper and better phytase enzyme available for use in formulated feeds. There is also evidence that the availability of rock phosphate may be less critical as a finite resource due the development of new reserves.

Therefore, for plant breeders the development of LPW is no longer attractive as a solution for the availability of phosphate to monogastric animals or for the reduction in phosphate pollution. However there is still interest in effects of LPW on the availability of certain micronutrients as the human health benefits could be quite significant in certain regions of the world.

3. TECHNICAL DETAIL

3.1. Introduction

3.1.1. The problem

A healthy plant contains 0.2–1.0% P in its total dry matter (Broadley *et al*., 2004), much of which occurs as polyphosphate and phytic acid salts (phytates) that are not used directly for plant growth (Marschner, 1995). In cereal grains, phytates form sparingly-soluble salts (with calcium, iron, magnesium, potassium and zinc) which have poor bioavailability in monogastric animals. Since phytates can contribute up to 80% of the total grain P, the P metabolism in plants can directly affect the dietary uptake of both phosphate and other essential mineral nutrients in monogastric livestock and humans. Understanding and modifying the phytic acid biosynthesis pathway in plants may offer a solution to a practical agricultural problem. Phytic acid participates in many different aspects of plant physiology.

Wheat can constitute up to 60% of pig and poultry diets in the UK. A variety of High Available Phosphate (HAP) wheat with similar performance characteristics to conventional wheat would ensure that the entire P requirement in the diet of monogastrics was met without the need to add supplementary phosphate. Ideally, it is anticipated that HAP wheat could also be used in conjunction with exogenous phytase to further reduce the release of P into the environment, since there is evidence to suggest that they work synergistically (Baxter *et al*., 2003). Varieties of maize and barley have already been produced with lower phytate contents and thus, correspondingly, HAP concentrations. Pigs fed with this HAP maize, reduced P excretion by 18% and by 16% when they were fed HAP barley (Veum *et al.,* 2001 and 2002; Spencer *et al.,* 2000).

The pig and poultry sectors in the UK have maintained their viability by maximising the economies of scale. In the near future under the Integrated Pollution Prevention and Control legislation and the EU Water Framework Directive, producers will be restricted from spreading muck on fields. This will threaten both the size and viability of the UK animal production industry and will significantly affect the animal feed supply chain. It has been demonstrated that some farmers fail to fully factor-in the contribution from manures when considering crop nutrient requirements and fertiliser input. This results in a considerable P surplus, estimated at 175,000t per year (Withers *et al.,* 2001; Chalmers *et al.,* 2001).

3.1.2. Aim of the project

The aim of this project was to provide adapted germplasm and tools for marker assisted breeding of HAP wheat**,** which could have the potential of significantly reducing diffuse P pollution when used in the diets of monogastric animals. In addition, the effect of P fertiliser treatment on the P

metabolism within the wheat plant and grain and on the grain composition of other important nutrients and micro-nutrients was to be determined.

3.1.3. Opportunities presented by the project

Both the arable and livestock farming environments of the UK are undergoing radical change: CAP review and the introduction of single farm payments and changes in legislation relating to diffuse pollution will have a profound effect upon the animal farming sectors and provision of animal feeds by the arable sector as well as their associated industries. There is concern that certain industries, notably outdoor pig rearing, will be affected more than most. In order for payments to be made, it will be necessary for land managers to comply with cross compliance rules, which includes keeping land in Good Agricultural and Environmental Condition (GAEC – Defra Cross Compliance Handbook 2005). A major facet of GAEC will be to keep accurate Soil Management Plans where both fertiliser inputs and the use of farm manures will have to be included. Furthermore, the development of a niche market for wheat produced for animal feed could help to maintain profitability in the light of decoupled payments. This may be through production contracts of HAP wheat varieties that could develop into both a UK and European market.

3.1.4. Technology base

There is extensive literature on the biology and agricultural value of low phytate barley and maize. There has, however, been no attempt in the UK to use low phytate wheat in monogastric animal feed. Furthermore, there is no germplasm available with the substantially reduced levels of phytate composition in the grain that would be needed to have a measurable impact on diffuse pollution problems associated with animal production systems. In the US, partners at the University of Idaho have invested considerable effort in a number of crops with reduced phytate content, most recently they have been concentrating on wheat.

The use of low phytate germplasm derived from GM technology is not currently an attractive option for the UK farming industry. It is, therefore, necessary for the breeding industry to develop material by conventional means if this trait is to be made available to the animal industry.

In the past, the control of diffuse pollution has not been a priority, however, new diffuse-pollution directives will take effect in the near future that will adversely affect the ability of farmers to maintain present stocking levels. The economic margin in animal production is already low and a reduction in stocking levels could lead to serious problems for many producers. This project sought to provide the necessary data and a model to determine how animals will respond to new feed formulations and the effect that this will have upon the local environment of a given farm or holding.

3.1.5. Potential scientific advances

A number of important advances are anticipated as a result of the work carried out in this project: this work aims to greatly elucidate both the underlying physiology and genetics of phytate production and its effect upon mineral nutrition and related development. As a result of feeding studies it has been possible to model the environmental impact of different feed formulations. This has important ramifications beyond the immediate objectives of this project as it could form the basis of decision support tools for policy making and management tools to facilitate compliance with pollution targets.

This project aims to increase our understanding of the genetics of phytate accumulation. This will allow the development of validated molecular markers allowing straightforward selection and introgression of the low phytic acid (*lpa)* trait into multiple backgrounds. These could be used alone or in combination with the existing germplasm to circumvent any unpredicted pleiotropic effects of individual mutations e.g. on straw strength.

3.1.6. Advantages for industry

Feed companies will benefit from the cost savings the improved nutritional value of these wheat types will offer and since mutagenesis-derived HAP wheat will be acceptable in other wheat-based feeds, there will be no issues relating to separation.

Plant breeders could benefit from producing these varieties as it may allow them to segment the market, which would only be supplied by UK adapted wheat varieties. It is unlikely that imports from other countries will meet the requirements of the feed companies and their customers in these new sectors for some time and this market lead may well result in significant exports particularly to Denmark and Holland where pig and chicken industries are already experiencing problems relating to diffuse pollution.

The model for production and marketing could be along the lines of the contracts offered by some leading bread bakers for specific varieties grown to defined production protocols using certified seed only.

3.1.7. Competitiveness of the UK arable industry

The pig and poultry industries may benefit directly from this project because it may allow them to meet anticipated pollution legislation whilst continuing to benefit from the economies of scale necessary for the profitability of their industries.

Farmers will benefit from the products of this project because it will allow them to exclusively deliver into a new and segmented market. It may be the case that these varieties could only be grown under contract, thereby, maintaining profitability for both the seed producer and the specialist farm producers. These markets may provide a regular demand for a product with a good price premium. It is also possible that Agri-environmental schemes, e.g. the Entry-Level Stewardship Scheme could be utilised to provide a mechanism to support the production and utilisation of low phytate wheat because they will have demonstrable environmental benefit.

3.1.8. Environmental benefit and impact assessment

This project has increased our understanding of the nutritional physiology of wheat lines with low phytate content and helps to quantify the contribution that conventional cereal varieties fed to pigs and poultry make to the P load from intensive units.

The environmental benefits will be to improve the P-use efficiency of UK agriculture in both the livestock and crop sectors. In the livestock sector, agricultural production accounts for the bulk of diffuse P pollution in the UK. The accumulation of P in the soil and water near highly intensive pig and poultry production units is an important environmental challenge and methods to reduce this wastage are needed. The environmental benefits of this research are clear; the widespread adoption of HAP wheat in pig and poultry diets will result in a substantial reduction (15–20% is a realistic estimate) in the amount of P excreted by pigs and poultry. This may reduce, by a similar order of magnitude, the amount of diffuse P pollution emanating from pig and poultry units if HAP wheat was commercialised.

In the crop sector, inefficient P fertilisation may lead to the excessive use of P fertilisers, which is costly for both farmers and for the environment. Almost 0.2% of the global energy budget is used in the manufacture, transport and application of P fertilisers, whilst the losses of P from agricultural land will also contribute significantly to the diffuse pollution of watercourses. Further, world reserves of rock phosphate, which are the primary source of P fertilisers, may be exhausted within the next few decades whilst 50% of all mined sulphur is used as sulphuric acid by the P fertiliser industry. The use of HAP wheat lines provides an opportunity for the sustainable production of a high-quality feed wheat which could be produced using lower fertiliser (or manure-based) P inputs. This work will integrate closely with Defra strategic R&D to improve the nutrient-use efficiency of crop production systems.

Objective(s)

There were 5 main objectives numbered.

Scientific objective(s)

- Produce the necessary tools (such as molecular markers) and germplasm with low phytate content for the plant breeding industry in the UK.
- Determine the effect on plant physiology of producing wheat with low phytate content; the effect on both P metabolism and other aspects of plant mineral nutrition will be considered.
- Determine the reduction in P excretion obtained by feeding pigs and poultry HAP wheat compared to conventional wheat.
- Model the likely changes in diffuse pollution if HAP wheat lines are widely used in UK farming.
- Delivery of results from the programme and relevant technology to the industry value chain.

The project was organized into 5 work packages:

- Work package 1: Effect of HAP wheat on phosphorus excretion in pigs and poultry.
- Work package 2: Development of a model for quantifying effect of HAP wheat on diffuse P pollution reduction.
- Work package 3: Development of germplasm.
- Work Package 4: Development of a toolkit for marker-assisted breeding of the HAP trait.
- Work Package 5: Determine the effect of P fertilizer treatment on the growth and phosphate metabolism of HAP wheat.

3.2. Work Package 1. Effect of HAP wheat on phosphorus excretion in pigs and poultry

3.2.1. Introduction

To determine the reduction in P excretion obtained by feeding pigs and poultry HAP wheat, feeding experiments were conducted comparing HAP with conventional wheat. In the first instance, it was decided that the most robust comparison would be between HAP wheat and the near isogenic line grown in the same location and conditions.

The two wheat samples (HAP and the near isogenic line) were then compared in a feeding trial in the presence or absence of exogenous phytase. The wheat samples were first tested before balanced formulated feeds were produced for the feeding trials. In the first trial, the effect of HAP wheat compared with conventional wheat on phosphorus availability in broilers was studied and used to inform further chicken and pig trials. Feed intakes, growth rates, feed conversion efficiencies and phosphorous balances were determined in each case. The University of Reading and Harper Adams University College undertook this work with technical assistance from Frank Wright, ABN and the MLC.

3.2.2. Production of the feed wheat

In 2005, 50kg of Lolo and the near isogenic line IDO0637 were provided by Idaho University for multiplication in the UK. In the LPA line Lolo, phytic acid P represents only 42% of seed total P, in contrast to 74.7% of seed total P in the IDO0637 control.

These US spring wheat lines were known to be very poorly adapted to UK growing conditions, due to poor disease resistance, the wrong dwarfing genes and little or no sprouting resistance. Therefore to overcome these problems it was planted very thinly later in the spring and with a full fungicide program. This produced a reasonable crop (very poor by UK commercial standards) as can be seen in Figure 1 with a total yield of approximately 750kg/variety of material after harvest.

Figure 1. Production of the US spring wheat, Lolo at NIAB Cambridge in 2005.

To provide sufficient material for both the planned chicken and pig experiments, a further multiplication was undertaken in 2006 to produce at least 20t of each line. However, the harvest in 2006 was very wet and significant sprouting had occurred in the material before harvest. Therefore, available phosphate analysis of the two lines after harvest showed little difference and the samples were of no use for the proposed feeding trials as the difference in the predicted available P content of the two lines would result in a smaller difference in excreta P content than the limit of detection in excreta P.

The process was repeated in 2007 and 20t with significant available phosphate difference were successfully produced. These were then made available to Reading University (1 t) and Harper Adams University (19t) for the feeding trial to commence.

3.2.3. Effect of LPW on phosphorus excretion of poultry

Two experiments were conducted to compare the availability of phosphorus in HAP wheat and conventional wheat (CON) for broilers. It was decided, in consultation with the project team, not to investigate phosphorus utilisation in laying hens but rather to concentrate efforts on broilers. This was because the broiler industry far outweighs the layer industry in the UK in terms of the numbers

of birds (844 million broiler chicks were placed in 2009 from UK hatcheries, compared with just 34 million layer chicks), and also because the higher calcium and phosphorus requirements of the laying hen for egg production compared with that of the broiler makes reductions in the inorganic phosphorus content of laying hen diets unlikely.

Experiment 1

In the first study to determine the effect of LPW compared with conventional wheat on phosphorus availability in broilers, three diets were formulated. These were formulated to be similar in composition to conventional wheat-based broiler grower and finisher diets and so a number of other ingredients (which contained phytate and non-phytate phosphorus) were added. The first, control diet was similar to a commercial broiler diet, and used the conventional(IDO367), near isogenic wheat that had been produced and contained 'normal' proportions of phytate-P. This diet was denoted CHP. The second diet, which contained the LPW, had the same calculated content of available P (based on an assessment of the availability of P in phytate and non-phytate P). This diet was denoted HAP. The third diet, based on conventional wheat, was formulated to have the same total P content as the HAP diet. This diet was denoted CLP. The rationale behind these diets was that the P content of the HAP diet had to be reduced (compared with CHP) as the higher availability of P in the HAP diet would result in surplus available P being fed, which may well be excreted so that there was no difference in the excretion of P between CHP and HAP. A positive control was therefore needed, in which the same total P content was fed; this was the function of CLP which had the same total P content as HAP. As the P in CLP was less available, however, more P was expected to be excreted.

170 day old (Ross 308) male chicks were purchased (PD Hook Hatcheries, Bampton, UK). On arrival, they were brooded together for two weeks in a single pen and fed a starter chick crumb. Clean, fresh water was always available. At 15 d their diet was changed to a grower diet with low phosphorus content (equivalent to the CLP formulation, Table 2). They were reared in a single pen for a further two weeks. At 28 d, they were weighed, blocked within groups according to weight and then randomly allocated to smaller pens (eight pens per treatment). The diet was changed to a finisher diet (and was CHP, HAP or CLP). Titanium dioxide was added as an internal marker to the grower and finisher diets. The composition of the diets used is presented in Table 2.

Table 2. Composition of grower and finisher diets (g feedstuff/kg diet, fresh weight basis).

Feed	Grower diet	Finisher diets		
	CLP	CHP	CLP	HAP
Fish- Provimi 66	10			
Soya (HiPro)	200	176	178	180

Extrupro (Beans)	40	40	40	40
AB Agri Starter/grower mineral mix	2.5	2.5	2.5	2.5
Lysine	2.83	2.72	2.64	1.99
Methionine	2.73	2.35	2.33	1.72
Threonine	0.69	0.72	0.68	0.20
Betaine	0.75	0.75	0.75	0.75
Grindazyme GPL 5000	0.50	0.50	0.50	0.50
Elancoban G200		0.50	0.50	0.50
Maxiban G160	0.63			
Limestone granules	10.57	8.93	10.87	11.31
Dicalcium phosphate	10.2	13.3	10.1	9.1
Salt	0.53	0.73	0.76	1.14
Sodium bicarbonate	2.48	2.48	2.45	1.90
Soya oil		7.76	3.56	2.92
Soya oil (spray)	21.4	9.68	14.2	14.6
Fat Hispec (veg) mixer	10.0	20.5	19.5	20.1
Fat Hispec (veg) spray		10	10	10
Control wheat	683	700	700	
HAP wheat				700
Titanium dioxide	1	1	1	1

Once the birds were allocated to their pens, feed intake was recorded on a daily basis. The amount of feed offered each day was recorded, as was the amount of feed refused the previous day. Feed refusals were cleared on a daily basis. A running sample of feed was made daily from days 28–42. Feeds were analysed for titanium dioxide and P.

Complete collections of excreta were made from each pen daily from day 28. The collections from days 28–35, and from 35–42, were bulked separately. Excreta samples were stored frozen before being mixed thoroughly, freeze dried, milled and stored at room temperature in air tight plastic containers. They were then analysed for titanium dioxide and P.

Birds were weighed when they were allocated to their treatments (at 28 d) and when they were slaughtered (at 42 d). The middle toe from the left foot was removed from each bird and analysed for ash content. The right leg was removed above the hock from one bird in each pen and sent to Roslin Institute for determination of bone strength.

The results of this first experiment are summarised in Table 3.

Table 3. Effect of wheat type and dietary phosphorus content on bird performance, phosphorus nutrition and bone strength

Parameter	Diet[1]			SEM[2]	P
	CHP	HAP	CLP		
Live weight gain (kg, 28-42 d)	1.88	2.03	1.94	0.0620	0.255
P content diet (mg/kg)	6045	5350	5760		
P availability	0.448	0.515	0.496	0.0119	0.001
P balance (g/bird/d)	0.474	0.543	0.487	0.0214	0.063
P excreted (g/kg bird weight gain)	4.40	3.75	3.99	0.241	0.186
Toe ash (g/kg)	113	105	108	2.3	0.054
Bone strength					
Max load (N)	235	238	238	19.1	0.989
Stiffness (N/m)	143702	135217	156076	15642.9	0.680

[1]CHP: a standard broiler diet using the conventional wheat. HAP: diet using the low phytate wheat (highly available P wheat), formulated to have the same available P content as CHP, based on estimates of total and phytate P content in the two wheat's. CLP: diet formulated using the conventional wheat, with the same total P content in the diet as the HAP wheat.

[2]SEM: Standard error of the mean

There was no significant effect of diet on bird performance. The availability of P was increased when diets HAP and CLP were fed. In the case of HAP, this could be a consequence of the increased availability of P because of the lower phytate content. However, since reducing the total P content of the diet (as was achieved with CLP) had the same effect, it is equally likely that the reduced supply of P made the utilisation of P more efficient. Although not significant (P=0.063), there was a tendency for P balance to be increased when HAP was fed, but there was also a tendency (P=0.054) for the bone ash content (as estimated by determining the ash content of the toe) to be reduced when HAP and CLP were fed, suggesting that P nutrition was more marginal in these diets. However, there was no evidence that bone strength had been affected by the P content or availability in the diet.

The interpretation of data from this experiment was complicated by the presence of other feeds, and other sources of P and phytate. The two wheats also had rather different protein contents (143 and 136 g/kg fresh weight for LPW and conventional respectively), so that the inclusion rates of other feeds in the diets were different to ensure the diets were isonitrogenous. It was therefore decided to conduct another experiment in which wheat was the sole component of the diet (apart from synthetic sources of amino acids, and additional minerals and vitamins) to determine what

effect changes in phytate content had on the availability of P in wheat. This was the rationale behind Experiment 2.

Experiment 2

Male chicks (160, Ross 308, PD Hook Hatcheries, Bampton, UK) were reared for five weeks as a single group. They were fed a proprietary chick crumb for 14 d, a proprietary grower diet from 15–27 d and a proprietary finisher diet from 28–35 d. The grower and finisher diets had titanium dioxide (TiO_2) added as an internal marker of digesta movement. At 35 d, the birds were weighed, blocked by live weight and grouped into smaller pens (twelve pens per treatment group, with six birds in each pen; there were a total of two treatments). The pens had solid floors with a bedding of wood shavings. The two treatments that were compared were diets consisting of ground wheat, with additional trace elements, salt, vitamins, minerals (with the exception of phosphorus) and amino acids (lysine, methionine and threonine) to balance the birds' requirements. Titanium dioxide was also added as an internal marker and the diets were pelleted. The wheat fed was either the wheat used in Experiment 1 with a low phytate content (LPW) or a conventional wheat, with an equivalent protein content to LPW (CON), (both diets comprised 966.5 g wheat/kg diet on an as fed basis).

The composition of the experimental wheat diets is presented in Table 4 and the composition of the vitamin/mineral mix in Table 5. From 38–42 d, a sample of each diet was taken (but not bulked with the previous day's sample). These samples of feed were analysed for titanium dioxide and P. Excreta were also collected daily from each pen by placing the birds in a separate enclosure (with no bedding) for one hour and collecting the excreta deposited in this time. Collections of excreta were bulked (by pen) with the previous day's sample. Excreta were thoroughly mixed, dried (105°C), milled (1 mm screen) and analysed for titanium dioxide and P. At 42 d, the birds were humanely slaughtered and a sample of ileal digesta was taken from each bird and bulked by pen. These bulked samples were further bulked by block (the four heaviest, the four of the second heaviest, and the four lightest, by treatment). Ileal digesta samples were mixed, dried (105°C), ground in a pestle and mortar and analysed for titanium dioxide and P. The left foot of each bird was also taken and bulked by pen. One foot from each pen was analysed for ash content.

Table 4. Composition of the experimental wheat diets (g/kg diet as fed)

Feedstuff	Diet	
	LPW	CON
LPW wheat[1]	966.5	
CON wheat[1]		966.5
Calcium carbonate	15	15
Lysine	7	7
Sodium chloride	3	3

DL methionine	3	3
Vitamin/mineral supplement	2	2
Threonine	2	2
Titanium dioxide	1	1
Tryptophan	0.5	0.5

[1]LPW: Low phytate wheat; CON: control wheat (conventional wheat with relatively high phytate content, compared with LPW).

Table 5. Composition of the vitamin/mineral mix used in the experimental wheat diets

Ingredient	Inclusion in premix (g/kg fresh weight)	
	LPW	CON
LPW wheat	921.5	
CON wheat		921.5
Choline chloride 50%	64.7	64.7
Manganese oxide 62%	3.9	3.9
DL-α -tocopherol acetate 50%	2.0	2.0
Zinc oxide 72%	2.8	2.8
Copper sulphate 25%	1.3	1.3
Nicotinic acid 99%	0.7	0.7
Ferrous sulphate monohydrate 30%	1.1	1.1
Selenium 1%	0.6	0.6
Cyanocobalamine 0.1%	0.2	0.2
97.5% calcium D-pantothenate	0.3	0.3
Vitamin A (retinol) 1 000 000 iu/g (0.01%)	0.2	0.2
Biotin 2%	0.1	0.1
Vitamin B_2 riboflavin (80%)	0.1	0.1
Vitamin D_3 cholecalciferol 500 000 iu/g	0.2	0.2
Menedione sodium bisulphite 51.5%	0.1	0.1
Pyridoxine hydrochloride 99%	0.1	0.1
Thiamine hydrochloride 99%	0.04	0.04
Folic acid 95%	0.03	0.03
Potassium iodate (59% I)	0.03	0.03

The availability of P in the small intestine and whole tract were calculated, and the ash content in the foot was determined. The effect of wheat type (LPW or CON) on these parameters was determined by analysis of variance. These results are summarised in Table 6.

Table 6. Effect of wheat type on the availability of P and ash content of the foot.

Parameter	Diet		SEM	P
	LPW	CON		

P content diet (mg/kg as fed)	4083	2760		
P availability:				
Small intestine	0.546	0.749	0.0177	0.001
Whole tract	0.540	0.748	0.0125	<0.001
Weight gain, 34-42 d (g/bird)	588	478	28.1	0.007
Foot ash content (g/kg dry matter)	114	115	2.4	0.961

The diet fed in this experiment was extreme in terms of its very high wheat content, and would not be suitable for commercial practice. The objective of the experiment had been to determine the availability of the phosphorus in the wheat, when the wheat had a low (LPW) or conventional (CON) phytate content. In a commercial diet, wheat would provide approximately 48% of total phosphorus. In these diets, all the phosphorus was supplied by the wheat, and so the birds had to utilise the phosphorus as efficiently as possible to meet their nutrient requirements. The CON wheat was selected on the basis of having a similar crude protein content to that of LPW (to overcome the problem in the first experiment; in which the two wheats had different protein contents so that the composition of the diets were different to allow for this). In this experiment, the protein contents of the two wheats were similar but the phosphorus content differed, with the CON wheat having a lower P content than LPW (approximately 68% of the P content of LPW). This lower dietary P content (and therefore P intake) with CON compared with LPW apparently increased the efficiency of utilisation of the dietary P, with the apparent availability of P in both the small intestine and whole tract being significantly greater with CON compared with LPW. The reduced P intake with CON (despite the increased efficiency of utilisation) could have had a detrimental effect on the growth rate of the birds and their bone strength (estimated from the ash content of their feet). Although the ash contents of the foot were very similar to the ash contents observed for toes in Experiment 1, and were not affected by wheat source, there was a significant effect of wheat type on bird performance. Birds fed LPW grew better between days 34 and 42 compared with those fed CON, achieving 23% more live weight gain. Thus, although there was no evidence to suggest that the birds fed CON were mobilising greater bone reserves of P compared with LPW, their growth rate was lower.

Conclusion

The results of these experiments confirm that P excretion by birds can be decreased by reducing the P content of the diet. The effect of dietary P content on the proportion of dietary P apparently excreted is summarised in Figure 2, using data from Experiments 1 and 2. Substituting CON wheat with LPW wheat did not further reduce the proportion of dietary P apparently excreted, as the LPW data points were not below the line of best fit. However, since a greatly reduced dietary P content negatively affects bird performance (and may indeed result in an increased P excretion per unit of live weight gain), there may be a role for LPW if more marginal P diets were fed (to reduce diffuse

P pollution from poultry units) to ensure sufficient P is available to meet the bird's requirements to maintain health and performance.

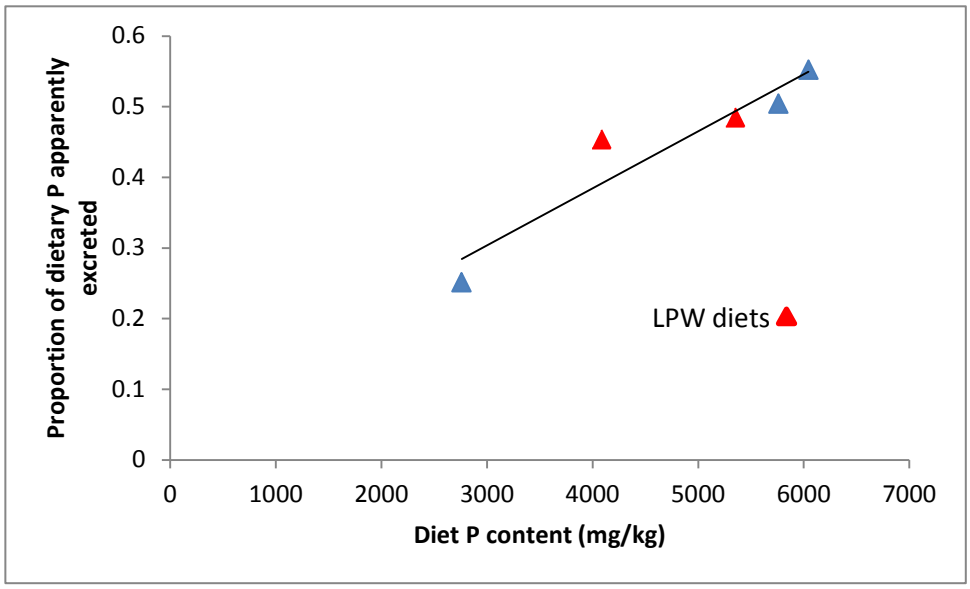


Figure 2. Effect of dietary P content on the proportion of dietary P apparently excreted by broilers

3.2.4. Effect of LPW on phosphorus excretion of pigs

Total pig output in the UK in terms of number of pigs is 9.5m/annum (down from 14+ m in 1998) and they consume 2.7m tonnes of feed comprising 50–55% wheat. It is estimated that 9,720 tonnes of P_2O_2 is excreted by pigs each year in the UK. The impact that low P availability has on the total P contents in commercial pig diets is illustrated in Table 7 as so much more is added than is actually required. Pig manure is generally utilised as efficiently as possible, however, where pig manure is spread, soil content of P has increased over a number of years. This is exacerbated by the 150,000 outdoor sows where waste output is diffuse across land that is uncropped for up to two years.

Table 7. The maximum potential loss of P from pigs to the environment in the UK.

	Kg feed per pig produced (MLC 2004)	Digestible P required g/kg (Whittemore *et al.,* 2003)	Total P supplied in typical diets g/kg.	Max. potential loss to the environment kg/pig
Sow feed	66.5	2.75	8	0.35
Weaner Feed	48.5	3.40	9	0.27
Finisher feed	168.0	2.40	8	0.94
Total	283.0			1.56

Following the removal of bone meal as a traditional source of P, there is now a greater reliance on adding phosphates in the form of dicalcium phosphate or the less digestible rock phosphates. An

increasing number of supplement manufacturers include phytase in pig diets, which results in a combination of reduced total P in the diet and increased P availability (up to 60% available across a number of studies; Jongbloed *et al* 2004, Pallauf *et al* 1993, Hoppe *et al* 1993). Inclusion of phytases have been shown to reduce faecal output of P by up to 30% (down from 625 g P/pig finished to 428 g P/pig finished). These data indicate that, while exogenous phytases can significantly reduce P excretion by pigs, there is scope, by the use of high available phosphate (HAP) wheat in the diet, to further reduce diffuse P pollution from pig enterprises.

Objectives

Using the original HAP seed sourced from the US (Lolo) and multiplied up in the first three years of the project, the objective was to establish the suitability of feeding low phytate wheat to pigs and enumerate the likely benefits. Early on in the project it was established from the literature that the variety of wheat would have little discernible impact on growth and performance and that Lolo could be treated as any other pig feed ingredient and used in standard formulation procedures within the constraints of the chemical analysis. The focus of the pig trials moved towards the precise value of improved P availability of using a low phytate wheat which could be applied to diet formulation in a range of commercial scenarios in order to assess the overall environmental and economic value.

The objectives were defined as:

1. To establish precise values for P availability where the wheat is presented in a meal or pelleted format in a commercial scenario.
2. To establish precise values for P availability in both finishing pigs and sows.
3. To confirm the likely effect on P availability by adding exogenous phytase.

Three digestibility trials were carried out using growing pigs and sows, housed in groups and fed a pure test wheat diet including an indigestible marker (Titanium Oxide (TiO_2)3g/kg). Faecal samples were then collected and the whole tract digestibility calculated.

Aims

The aim of this trial was to establish the digestibility of phosphorous between the two wheat varieties of normal and high phytate content and identify the effect of the high temperature processing used in the manufacture of pellets on the activity of the naturally occurring phytate. The choice of wheat was limited to 4 samples produced over two harvests. They were matched as close as possible for protein content, which were both relatively high, and phytase which were very similar given the natural variability.

Trial 1: Evaluation of P availability in finishing pigs Objective 1&2.

A total of 160 pigs were selected over two time periods (20 pens of 8 pigs at 25+ kg of mixed sexed). They were acclimatised for 2 weeks on an ≈80% wheat based diet using a commercial grower formulation 25–45 kg (CP 17.6%, lys 1.11%, oil 4.0). They were housed on solid floored pens and bedded on wood shavings. All pigs were of a modern commercial genotype sourced from PIC and JSR. The treatments were balanced for sex and breed. At 40 kg, all bedding was removed from the pen and the pure wheat based test rations were introduced to provide a minimum of maintenance requirements. It was based on 96%+ wheat and nutritionally balanced where possible using non-P ingredients (ie oil, amino acids and vitamins and minerals) + TiO_2 inert marker at 3g/kg (Appendix B). The test diets were fed freely on a pen basis with feed intake recorded over the 10 day period. Pens had the bedding removed at this stage to avoid any additional P entering the digestive system. Manipulable toys were provided to enable more natural behaviours. The pigs were acclimatised to the diet for seven days (Jagger *et al.,* 1992) and a faecal recovery method with minimum interference was used (Thacker *et al.,* 2003) over a period of three days. Pigs were removed from the pen twice a day (for up to 30 minutes) to a specially prepared pen where faeces were recovered from the floor with minimum contamination. Staff were present at all times to ensure a good faecal recovery (the time was variable depending on the pig). Diet samples were taken from each pen during the collection period and analysed for dry matter, nitrogen, phosphorous, phytase activity and TiO_2. Faecal samples were pooled by pen on a daily basis and sub-sampled giving a total of 20 feed and 60 faecal samples.

Treatments

There were four wheat treatments in a 2x2 factorial design (5 pens per treatment):

- Two wheat varieties were used Lolo HAP wheat from the 13/8 harvest date and a control from the 30/8 harvest date. The control variety (IDO 367) was a second US wheat variety of similar phosphorous and protein content to match the relatively high protein content of Lolo (Table 8).
- The diets formulated using the two wheats were fed either as a meal (no heat treatment), or a high temperature treated pellet.

Table 8. Preliminary check analysis of candidate wheats

	Protein %	Calcium %	Phosphorus %	Phytate	Phytase
LOLO 13/8	14.11	0.09	0.36	0.13	570
IDO 30/8	15.11	0.11	0.35	0.27	590

Results

The high temperature pelleting process resulted in temperatures at the die and pellet conditioner of 91.5°C and 88.5°C, respectively. This had the desired effect of reducing the endogenous phytase

activity to a very low level which would be expected in standard commercial diets that are not supplemented with exogenous phytase. Feed intake and performance was unaffected by treatment and there were no abnormal health issues recorded in relation to treatment. Statistically two of the individual replicates in the Titanium analyses were considered outliers and omitted from the analysis.

Protein digestibilities were as expected and did not vary between treatments. The small, but significant, difference in protein content of the wheat (Table 10) seemed to have little impact on the overall digestion process. There were no obvious detrimental effects on the growth.

Temperature/pelleting treatment reduced the endogenous phytase activity as expected; however, this was not reflected in the P digestibility between processing types where there was no significant difference (Table 10). There was a 12.3% difference in digestibility of P in HAP wheat compared to the control which resulted in a 19% reduction in faecal output of P. There was an indication that where endogenous phytase is present, there is an additive effect of using a high available P wheat (Table 9).

Table 9. Trial 1 Interaction Analysis

Wheat Treatment	**HAP**		**Control**		**Sed**[1]	**P value**
Feed Format	**Meal**	**Pellet**	**Meal**	**Pellet**		
Diet Analysis						
Protein (%)	14.1	14.2	15.1	15.4	0.080	0.068
Phosphorous (%)	0.35	0.37	0.37	0.38	0.017	0.516
Phytase Activity (FTU/kg)	588	126	512	72	13.90	0.281
Feed intake on test (kg/day)	1.03	0.95	1.11	1.11	0.094	0.551
Nitrogen Digestibility (%)	72.6	74.3	73.5	75.2	1.990	0.996
Phosphorous Digestibility (%)	**39.7**	**36.3**	**30.6**	**20.9**	**6.30**	**0.486**
Faecal P Content (%DM)	1.14	1.39	1.36	1.75	0.067	0.166

1 Standard error of the difference

Table 10. Trial 1 Treatment Factor Analysis

	Wheat				**Feed Format**			
Treatment	**HAP**	**Control**	**Sed**	**P value**	**Pellet**	**Meal**	**Sed**	**P value**
Diet Analysis								
Protein	14.2	15.3	0.050	<.001	14.8	14.6	0.050	<.001
Phosphorous	0.36	0.37	0.012	0.334	0.37	0.36	0.012	0.262
Phytase Activity (FTU/kg)	357	292	9.900	0.001	99	550	9.900	<.001
Feed intake on test (kg/day)	0.99	1.11	0.067	0.083	1.03	1.07	0.067	0.557
Nitrogen Digestibility (%)	73.4	74.3	1.400	0.515	74.8	73.1	1.400	0.245
Phosphorous Digestibility (%)	**38.0**	**25.7**	**4.430**	**0.014**	**28.6**	**35.1**	**4.430**	**0.159**
Faecal P Content (%DM)	1.26	1.55	0.047	<.001	1.57	1.25	0.047	<.001

Trial 2: Evaluation of P availability in sows Objective 2&3.

A total of 16 multiparous, non-pregnant sows were selected in 2 batches of 8 from at least 14 days post weaning (233kg (sd 19.5)). They were housed in groups of 8 and fed individually once a day in standard commercial feeding crates. The pens were bedded with wood shavings and the sows were acclimatised to a standard commercial dry sow ration (Oil: 5.75, CP: 13.00, Fibre: 6.5, Ash: 6.0, Lysine: 0.6) to a point where they were a body condition score of at least 3 (on the standard commercial scale of 1–5). At the start of the trial, sows were weighed, back fat measured and assigned to one of four treatments. Each sow then progressed through each treatment in a Latin square design. Each treatment was based on the same wheat types and formulation as in trial 1 (Appendix A) with a vit/min pack designed for sows and a TiO_2 marker at 3g/kg. The diets were fed individually with a target of 2.25kg per day in a single meal. Sows were fed in individual feeders and remained in the feeder for a total of 30 minutes at each feeding time. The sows were acclimatised to the treatment diets for at least seven days (Jagger et al., 1992) and faecal recovery was done over a further 3 days. Faecal recovery was made at the end of the confined feeding period in the morning with minimal interference. Triplicate diet samples were taken from each treatment during each collection period and analysed for dry matter, nitrogen, phosphorous, phytase activity and TiO_2. Faecal samples were pooled by individual sow over the three days of collection. This resulted in 16 faecal samples and 6 feed samples per treatment.

Treatments

There were four wheat treatments in a 2x2 factorial design (16 sows per treatment):

- As in trial 1, two wheat varieties were used Lolo HAP wheat from the 13/8 harvest date and a control from the 30/8 harvest date.
- The above wheats were fed with or without the addition of exogenous phytase, Natuphos®

All the diets were pelleted at a standard temperature (die temp range 58–68ºC) as this is the standard delivery method of feed in the UK. The exogenous phytase is a heat stable product that over comes the negative effect of pelleting on endogenous phytase as seen in trial 1.

Results

Diet analysis was more variable than in the first trial with a significant interaction in protein content between the two factors (Table 11). A second analysis was made on the feed samples with the same overall differences. The pelleting process had the desired effect of reducing endogenous phytase activity resulting in a significant difference between the diets with and without the exogenous phytase (Table 12). The feed intake was restricted to 2.25 kg, however, there were refusals with one sow being replaced early in the trial, resulting in a slight numerical difference in overall intake between the wheat types. There were small changes in weight and back fat over the four time periods (Table 13).

For this trial the digestibility of the feed nutrients are presented as proportions of intake (Tables 11&12). At between 10% and 14% excreted, the digestibility of nitrogen was consistent and high, perhaps suggesting that the diets were limiting. The level of faecal P indicated that there were endogenous losses from body tissue given that the output was the same or greater than intake (Table 12). There were no obvious reasons for these losses although the sows were not increasing in weight and the nutrient intake was likely to be close to maintenance. Endogenous losses are often attributed to a change in gut microflora and gut lining losses when diets are changed. Table 13 shows that during the first period of treatment, sows generally lost weight. When this data was excluded from the analysis (Table 14) there were still inexplicable losses. When daily live weight gain was used as a covariate there was no effect on the treatment means.

Both the wheat and exogenous phytase treatments had a significant positive effect on the relative output of phosphorous which showed a similar result to trial 1 where in this experiment, there was an additive effect of using both high available phosphorous wheat and reduced endogenous phytase activity. In this trial, the effect of phytase was greater than that of the wheat, highlighting the fact that the exogenous phytase used was relatively robust.

Table 11. Trial 2 Interaction Analysis

Wheat Treatment	**HAP**		**Control**		**Sed**	**Sig**
Exogenous Phytase	**+**	**-**	**+**	**-**		
Diet Analysis						
Protein	16.8	14.2	15.2	15.5	0.330	<0.001
Phosphorous	0.37	0.30	0.29	0.28	0.036	0.229
Phytase Activity (FTU/kg)	830	162	948	180	93.200	0.463
Feed intake on test (kg/day)	2.0	2.0	2.26	2.23	0.175	0.892
DLWG during test (g/d)	160	218	15	149	246.000	0.827
Avg. Daily Back Fat depth Change (mm)	-0.044	0.034	-0.061	-0.090	0.056	0.178
Faecal Nitrogen as (%) of Intake	11.0	13.7	10.3	11.9	0.980	0.453
Faecal Phosphorous as (%) of Intake	90.5	108.7	101.0	128.7	10.200	0.516

Table 12. Trial 2 Treatment Analysis

	Wheat				**Phytase**			
Treatment	**HAP**	**Control**	**Sed**	**Sig**	**+**	**-**	**Sed**	**Sig**
Diet Analysis								
Protein (%)	15.5	15.4	0.230	0.613	15.9	14.8	0.230	<0.001
Phosphorous (%)	0.33	0.29	0.025	0.083	0.33	0.29	0.025	0.149
Phytase Activity (FTU/kg)	496	564	65.900	0.326	889	171	65.900	<0.001
Feed intake on test (kg/day)	2.0	2.24	0.120	0.046	2.13	2.11	0.120	0.880
DLWG during test (g/d)	189	82	174.000	0.543	87	183	174.000	0.585
Avg. Daily Back Fat depth Change (mm)	-0.005	-0.076	0.040	0.078	-0.053	-0.028	0.040	0.527
Faecal Nitrogen as(%) of Intake	12.4	11.1	0.690	0.072	10.6	12.81	0.690	0.003
Faecal Phosphorous as (%) of Intake	99.6	114.8	7.210	0.039	95.7	118.7	7.210	0.002

Table 13. Trial 2 Sow body condition changes over the four treatment period within the Latin square design.

Period	**1**	**2**	**3**	**4**
Feed intake on test (kg/day)	2.00	2.02	2.31	2.15
Av Daily Live Wt Change (g/d)	-203	149	52	543
Av Daily P2 Change (mm/d)	-0.020	-0.080	-0.060	0.001

Table 14. Trial 2 Treatment Analysis (excluding period One)

	Wheat				**Exogenous Phytase**			
Treatment	HAP	Control	Sed	Sig	**+**	**-**	Sed	Sig
Faecal Nitrogen as (%) of Intake	12.6	11.3	0.85	0.149	10.8	13.1	0.85	**0.008**
Faecal Phosphorous as (%) of Intake	101.1	119.5	8.61	**0.038**	100.2	120.4	8.61	**0.024**

Trial 3: Evaluation of P availability in finishing pigs Objective 1&3.

In the final trial, the use of the high available phosphorous US wheat was compared to a UK sourced wheat processed in a standard way using exogenous phytase. This was done in growing pigs using the same protocol as trial 1. A total of 160 pigs were selected over three time periods (20 pens of 8 pigs at 25+ kg of mixed sexed). They were acclimatised for 2 weeks on an ≈80% wheat based diet using a commercial grower formulation 25–45 kg (CP: 17.6%, Lys: 1.11%, Oil: 4.0%). They were housed on solid floored pens and bedded on wood shavings. The treatments were balanced for sex and breed type. At 40 kg, all bedding was removed from the pen and the

pure wheat based test rations were introduced to provide a minimum of maintenance requirements. It was based on 96%+ wheat, nutritionally balanced where possible using non-P ingredients (ie oil, amino acids and vit and min) + TiO_2 Inert marker at 3g/kg (Appendix A). The test diets were fed *ad lib* on a pen basis with feed intake recorded over the 10 day period. The pigs were acclimatised to the diet for seven days (Jagger et al 1992) and a faecal recovery method with minimum interference was used (Thacker et al 2003) over a period of three days. Feed samples were taken from each pen feeder during the collection period and analysed for DM, nitrogen, phosphorous, phytase activity and TiO_2. Faecal samples were pooled by pen on a daily basis and sub-sampled giving a total of 20 feed and 60 faecal samples (3 pen samples per replicate).

Treatments

There were four wheat treatments in a 2x2 factorial design (5 pen reps per treatment):

- Two wheat varieties were used, Lolo HAP wheat from the 13/8/2007 harvest date and a new UK control from the 2009 harvest (normal commercial sample). The control sample was selected on the basis of a relatively high protein content to match the Lolo wheat previously used.
- The above wheats were fed with or without the addition of exogenous phytase, Natuphos® at an inclusion of twice the standard rate used in trial 2.

All the diets were pelleted at a similar standard temperature to trial 2 (die temp range 58–67ºC).

Results

The control wheat sourced from the UK was a high protein sample from the commercial stocks available, however it was lower than the US control used in the previous studies. More importantly, the total phosphorous content of this wheat was 0.2% which was 0.14% lower than the Lolo HAP wheat (Table 15). This figure is below the recommended 2.75 g/kg in finisher feed (Whittemore *et. al.* 2003). As expected the phytase activity was higher in the supplemented treatment compared to trial 2. The endogenous phytase was high compared to the previous studies, perhaps indicating a low pelleting temperature.

There were no obvious signs of ill health and loss in performance. Gut health was maintained with nitrogen digestibilities in the same range as in trial 1 (70–75%). There were no treatment effects on the nitrogen digestibility indicating that the reduced protein content of the control had little impact on the overall efficiency of digestion.

The phosphorous digestibility in the untreated HAP wheat (Table 15) had a similar value to the same treatment in trial 1, indicating that there is a good repeatability. The addition of phytase had a significant effect on the P digestibility of an extra 12% (Table 16). The effect of the HAP wheat is

less clear. There was no overall wheat treatment effect; however, there is a numerical interaction between the factors. Within the supplemented treatment there was a lift in P digestibility of 6%. This, however, is not observed in the non-supplemented treatments. One possible reason for this is the nutritionally low level of total P in the UK wheat exerting a greater pressure on the digestion of P in this sample. Also at low levels of total P there is less of an effect of using phytase. To try and account for this, total feed P was included as a covariate which adjusted the means for P digestibility in the expected direction but not enough to be able to draw firm conclusions (Tables15&16).

Table 15. Trial 3 Interaction Analysis

Wheat Treatment	**HAP**		**UK Control**		**Sed**	**Sig**
Exogenous Phytase	**+**	**-**	**+**	**-**		
Diet Analysis						
Protein (%)	14.5	14.5	13.4	13.7	0.180	0.179
Phosphorous (%)	0.33	0.35	0.19	0.21	0.005	1.00
Phytase Activity (FTU/kg)	1783	553	1923	703	58.60	0.907
Digestability analysis						
Nitrogen Digestibility (%)	73.9	71.3	73.3	74.3	3.060	0.417
Phosphorous Digestibility (%)	50.6	32.3	44.7	39.0	6.210	0.166
Phosphorous Digestibility (%) Adjusted for Total P content	50.6	33.4	43.4	37.1	6.140	0.221

Table 16. Trial 3 Treatment Analysis

	Wheat				**Phytase**			
Treatment	**HAP**	**Control**	**Sed**	**Sig**	**+**	**-**	**Sed**	**Sig**
Diet Analysis								
Protein	14.5	13.5	0.130	<0.001	13.9	14.1	0.130	0.354
Phosphorous	0.34	0.20	0.004	<0.001	0.26	0.28	0.004	0.002
Phytase Activity (FTU/kg)	1168	1313	41.500	0.008	1853	628	41.500	<0.001
Nitrogen Digestibility (%)	72.6	73.8	2.170	0.575	73.6	72.8	2.170	0.709
Phosphorous Digestibility (%)	41.5	41.8	4.390	0.931	47.6	35.7	4.390	0.015
Phosphorous Digestibility (%) Adjusted for Total P content	40.3	42.0	4.340	0.694	47.0	35.3	4.340	0.016

Conclusions from pig feeding trials

This set of 3 trials clearly shows that the use of a high available phosphorous variety of wheat can have benefits in increasing the digestibility of P by between 6 and 12 % points above the effect of

added phytase. This can reduce faecal phosphorous by up to an additional 20%. An increase in digestibility of 10 % points is equivalent to 2250 tonnes of phosphorous that the UK pigs would not need in the diet, nor excrete.

In formulating diets, other factors that need to be considered are the stage of production and stock category and their requirements, the format of feed delivery and the level of exogenous phytase. There was no evidence of a HAP wheat having any detrimental effects on animal health and performance other than the issues associated with correct feed formulation. In future variety selection programs, total phosphorous needs to be considered alongside the level of phytate and endogenous phytase.

3.2.5. Work package 2. Development of a model for quantifying effect of HAP wheat on diffuse P pollution reduction

Introduction

To inform policy and the selection of appropriate strategies in different situations a suitable model would allow the benefits of feeding HAP wheat to be evaluated. The data from work package 1 has been used to evaluate the likely benefits from using HAP in the UK agricultural environment relative to the other sources of phosphate pollution.

Model description

This has been presented (not published) as an abstract paper for submission to journals for publication. The full abstract is attached in Appendix B of this report.

Conclusions (see tables and references in the text Appendix B)

Dietary studies suggest that pigs and poultry fed cereal grains with low phytate concentrations excrete between 10 and 43 % less total P than those fed conventional varieties (e.g. Spencer *et al.*, 2000; Veum *et al*., 2001, 2002; Baxter *et al*., 2003; Jang *et al.,* 2003; Toor *et al.,* 2005; Leytem and Maguire, 2007; Leytem *et al*., 2007). Assuming that feeding pigs and poultry with low phytate cereals reduces total P in their excreta by 20%, it can be calculated that by replacing conventional feed with a low phytate alternative, it would be possible to reduce the total P load to GB waters by 0.53% (321 tonnes P/yr and the agricultural contribution to the total P load to GB waters by 2.73% (Table 1). These are small, but significant, reductions. If, in addition, total P loads from industrial fish farming, which contributes at least 963 tonnes/yr of total P to GB waters (White and Hammond, 2009), could be reduced by 20%, this would represent a further reduction of 0.32% to the total P load to GB waters.

Human sewage makes a major contribution to the total P load to GB waters (Table 1; White and Hammond, 2009). Much of this is derived from human excreta (Smith et al., 2005). Cereals

contribute about 31% of the energy input to human diets in GB (Hoare et al., 2004). If humans also ate low-phytate cereals, and the P in their excreta was consequently reduced, this could have a large impact on the total P load to GB waters. However, any reduction in the amount of phytate in human diets should proceed with caution because high dietary phytate has been linked to various health benefits (Vucenik and Shamsuddin, 2006). It is, therefore, not very likely to happen.

3.2.6. Work package 3. Development of Germplasm

Introduction

The pig and poultry feeding trials were conducted using germplasm developed by the University of Idaho. Multiplication in the UK was very problematic and served to illustrate the required level of adaptation needed to make the trait a commercial reality within the UK. Development of UK adapted material was itself a major undertaking and required a significantly longer time period than the duration of this 4 year project. Therefore the feeding trials were undertaken using non-adapted material.

Three types of material were developed in this work package, for three distinct purposes:

1) Trait introgression. Adaptation of the Idaho LPA line.

The University of Idaho selected the line Lolo from a mutagenised commercial line IDO0637. In the LPA line Lolo, phytic acid P represents only 42% of seed total P, in contrast to 74.7% of seed total P in the spring-sown control. This resulted in a greater than 33% increase in the phosphate that would be available to monogastric animals. However the Lolo line was unadapted to northern European growing conditions and needed to be adapted using a backcrossing programme with selection.

At this stage we appreciated that the new LPA trait would be most valuable to UK plant breeders if it was in an elite plant breeding background. Material that was commercially competitive in the UK in 2004 would already have been outdated by the end of any backcrossing program. Therefore, on the 16/09/2004, Limagrain provided us with their ten best pre-national list candidates. We hoped that at least one line would become a fully recommended variety and by that time we would have a LPA adapted equivalent.

Table 17. Material supplied by Limagrain

Line Number	Breeding code	Commercial name
1	A50 – 03	
2	A55 – 03	
3	A45	Zebedee
4	A51 – 03	

5	A53 – 03	
6	A43	Asagai
7	A41	Piranha
8	A41 – 02	
9	A42 – 02	Director
10	A46	Gatsby

Due the relative exotic nature of the Idaho material we anticipated that it would be necessary to reduce the Idaho background to at least 25% or less of the genetic component. To be sure of maintaining the trait of interest we would therefore need to screen all the material produced from and after the BC1 generation. When any of the lines were withdrawn from the National List or Recommended List process, the crossing with that line was stopped. By 26/6/2006 we were only working with the lines 1, 3, 4 & 9. This was eventually reduced to the line 3 which became Zebedee.

The following crossings were completed as indicated in Table 18. However due to the limitations of the selection method and the need to select for more than one gene the trait proved difficult to follow (section WP3-4 covers the details of the wheat flour colorimetric Pi test). This resulted in the need for additional crossing and testing.

Table 18. Crossing schedule

	Date when crossing started	% Lolo / UK line
Cross	16/9/2004	50/50
BC1	10/06/2005	25/75
BC2	20/05/06 – half seed analysis	12.5/87.5
BC3	13/2/07 – half seed analysis	6.25/93.75

The BC3 material was selfed in a polytunnel to produce larger amounts of seed for field sowing in the following autumn.

Figure 3. Multiplication of BC3 material in the summer of 2007 for field sowing in the autumn.

The 2008 field-sown material allowed large amounts of seed to be tested but the result was very disappointing. It suggested that some of the original crosses had been made with the wrong lines. This had concerned us for some time due to the limitations of the selection method possibly giving false positives. However, we had to do the crosses to run the testing. As tests were made at each generation it will be necessary to go back at least one generation. This illustrates the need for a more reliable marker based selection system.

However, in 2006 we had gone back to an original BC1 cross and selfed all the progeny. This allowed us to test more than half a seed and replicate the test. Consequently the BC2 seed that was produced in 2007/08 was considered to have a greater chance of carrying all genes of interest. These were again selfed by growing in a polytunnel which allowed a large quantity of seed to be produced for field sowing in 2009.

Figure 4. BC2 nursery in 2009 at NIAB.

All the 2009 field sown material was tested using residual seed from the polytunnel.

Ten plants within the best 20 lines were also leaf sampled and tested using the first markers that we had developed (section WP4).

Although all the nursery material was at least 87.5% of Zebedee genetics there was some significant variation in the nursery, see Figure 5. It was, therefore, possible to discard poor material and select some of the better types.

Fig 5. Variation in the BC2 Zebedee lines.

Single plants from the best 14 lines were sent to Limagrain UK Ltd (formally Advanta Seeds) for further field evaluation and multiplication in 2009/2010.

The residual seed was retested at NIAB using the wheat flour colorimetric Pi test but we failed to find the LPA lines. We are currently re-running all this material and will return to the earlier crosses

if we have lost the trait. This again highlights the need for markers to make accurate selections and allow the plant breeders to breed commercial lines.

2) Development of a DH mapping population for the Js-12-Mu-6 HAP wheat.

This work was undertaken to facilitate the objectives of WP4.

Lolo was crossed with the UK spring wheat Cadenza to produce more than 200 F1 seeds which were sent to Limagrain UK Ltd, for double haploid production. To ensure that the crosses had been successful the seed was checked to ensure that they were not selfed.

A total of 248 DH lines were produced and these were multiplied to produce enough seed for evaluation. There were three stages of evaluation:-

1. Seed was characterised for phosphate availability using the wheat flour colorimetric Pi test.
2. DNA was extracted from leaf samples and analysed by microsatellite markers linked to possible candidate gene locations. DNA was then subsequently sent for DArT analysis.
3. The remaining seed was grown at the Limagrain plant breeding station in Docking, Norfolk to characterise the progeny in the field. Notes were taken for disease and value score.

Figure 6. Low phytate wheat DH line 138, July 17th 2009.

The complete picture set is available from the NIAB picture library.

A total of 248 DH lines were examined for available free phosphate content of grain using the colorimetric assay. Microsatellite mapping was targeted to the three chromosomal regions where the candidate genes had been located by physical mapping. Initial quantitative trait loci (QTL)

studies revealed very small effects on these linkage groups, which was due to poor quality phenotypic data.

The colorimetric assays were then repeated, as was the QTL analysis using the composite interval mapping (CIM) function in QTL Cartographer. Two QTL were identified, which explained 25 and 32% of the variation in the colorimetric data. In addition, there was also an epistatic interaction between the two QTL accounting for a further 9%, giving the total amount of variation explained as 66%.

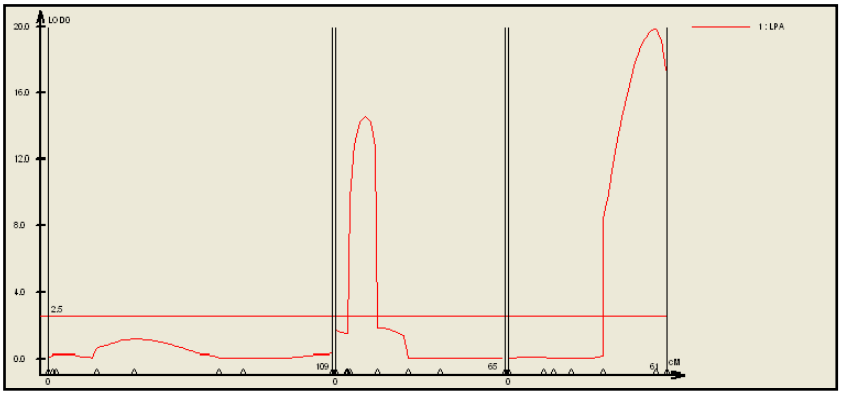


Figure 7. QTL Cartographer

We were concerned that some of the DH lines appeared to contain a higher level of iP than the *lpa* mutant parent and wanted to test whether any other QTL remained to be identified, possibly having come from the Cadenza parent. DNA from 186 DH lines, plus the parents', was sent for DArT analysis. A new linkage map was constructed in MapDisto using the marker data from 183 lines, which comprised 33 linkage groups encompassing 720 loci (27 SSRs, 693 DArT loci). A total of 24 loci remained unlinked. A subset of 269 evenly-spaced loci were used for QTL analysis and below are the results for just the three chromosomes with significant QTL effects on free phosphate seed levels (Figures 8–10).

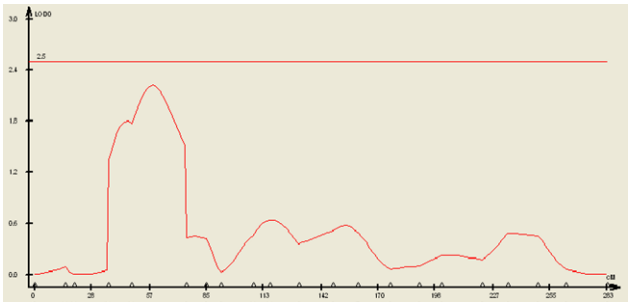


Figure 8. Chromosome X

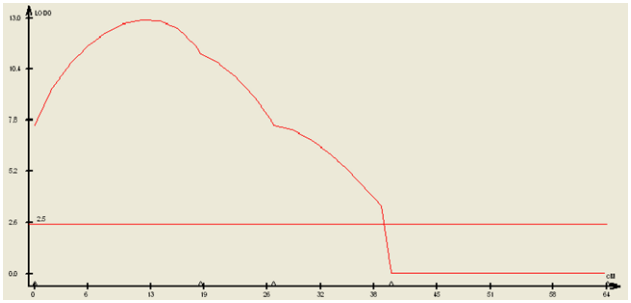


Figure 9. Chromosome Y

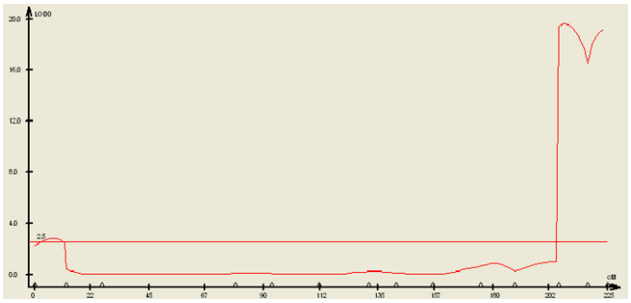


Figure 10. Chromosome Z

We have identified major QTL from the *lpa* mutant line in the DH population. These are co-located in the genomic regions where genes in the phytate pathway map. We are currently not sure if there are one or two QTL on chromosome Z due to the lack of markers as the region is “off the end” of the known SSR (simple sequence repeat) genetic map.

The SSRs linked to the QTL on chromosomes Y and Z are not diagnostic enough to follow the low phytate trait in a commercial breeding programme because they are not in linkage disequilibrium with alleles at the QTL. We therefore still need to find the single nucleotide polymorphisms (SNPs) in the candidate genes themselves (perfect markers), which we hope will come through sequencing bacterial artificial chromosomes (BACs) corresponding to the genes which co-locate with the QTLs.

3) Development of an EMS mutant population.

We originally proposed to develop our own ethyl methanesulfonate (EMS) population using the variety Einstein to discover alternative sources of mutations in the phytic acid pathway, which might be different to those in the Lolo material. However, for practical considerations we decided to use the publicly available EMS population from JIC which was generated as part of the WIGIN project. The mutant population was generated in Paragon and was available as an M6 generation, which would be an acceptable starting point for introgression of any mutations identified into Limagrain breeding material.

Over 1200 individual lines from the Paragon M6 seed stocks held at JIC were assayed using a crude estimation from a single ground seed/line in the wheat flour colorimetric Pi test. The samples which lay at the top of the distribution (110 lines) were then re-assayed with duplicate samples taken from 5–10 seeds milled into flour.

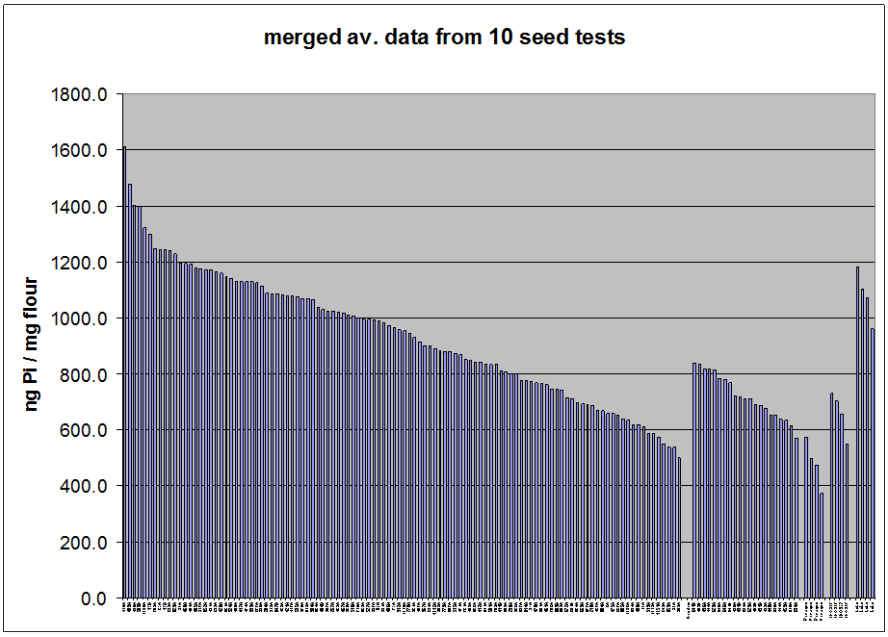


Figure 11. Paragon EMS population screen.

A total of 28 lines were chosen after further analysis, grown at NIAB and re-tested rigorously. These 28 lines consistently demonstrated a higher availability of iP by the colorimetric method, and progressed into the backcrossing program.

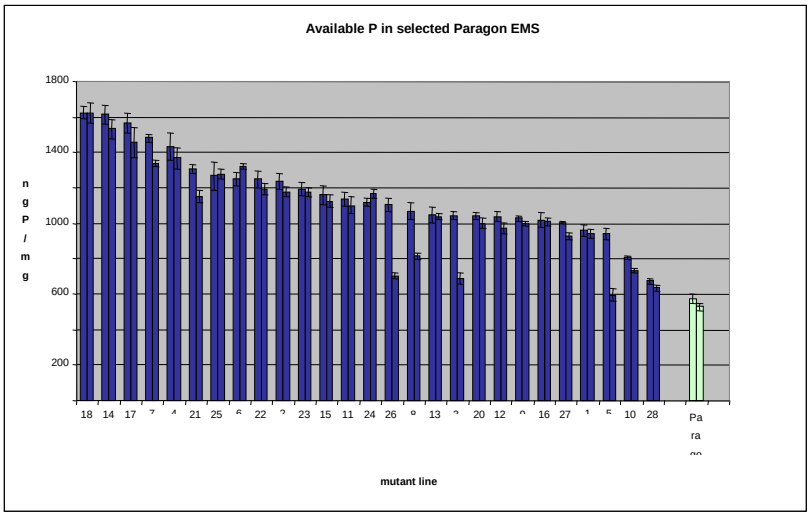


Figure 12. Selected material for further crossing.

Selected EMS Paragon lines were kindly analysed by Alex Murphy (University of Cambridge) for InsP6 and InsP3/4/5 intermediates by HPLC.

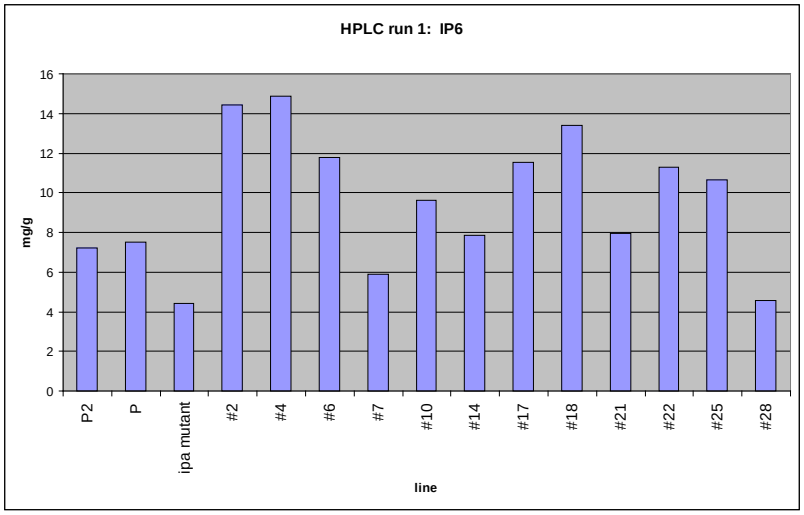


Figure 13. InsP6 and InsP3/4/5 intermediates by HPLC.

HPLC analysis appeared to show that two lines (7 and 28) were substantially reduced in their levels of IP6 compared to Paragon controls (P, P2). Many of the lines demonstrated a significantly higher level of IP6. This could result from differences in growth of the controls and mutants as unfortunately it was not possible to supply control material which had been grown at the same time as the mutant lines. An alternative explanation is that these lines have mutations in phosphate metabolism rather than phytate biosynthesis and that the levels of iP and IP6 are elevated.

A subset of 12 lines were also analysed for micronutrient analysis by ICP-OES (Martin Broadley, Nottingham). The preliminary results appear to show higher total P levels, which correlated with elevated Mg and K. However, again, we have the complication that it was not possible to supply control material which had been grown at the same time as the mutant lines.

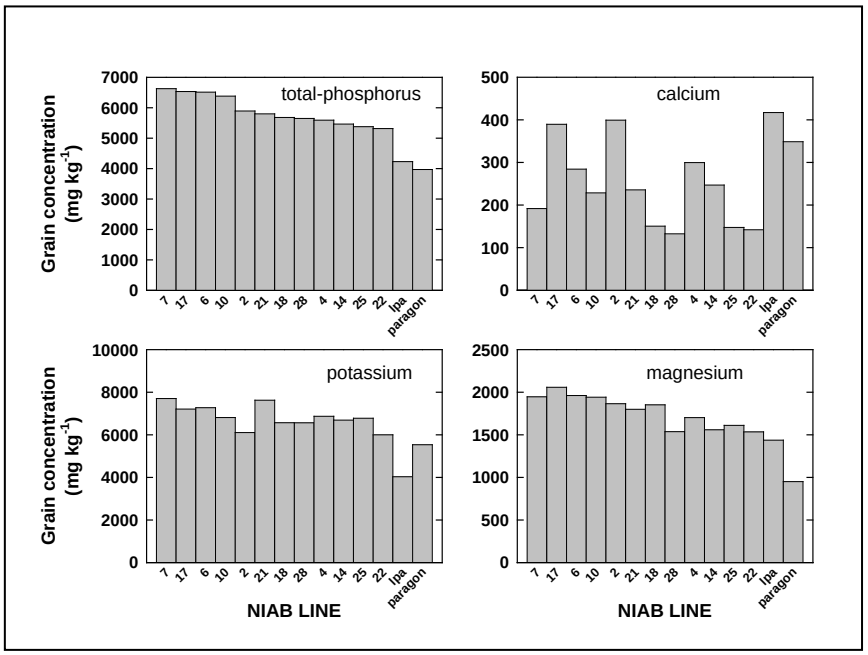


Fig 14. Micronutrient analysis by ICP-OES

The material and controls were therefore grown at the same location by the University of Nottingham for analysis. The results suggested that additional phosphate had been accumulated by all of these lines relative to the Paragon control. This suggests that mutations may have occurred in other areas of phosphate metabolism than currently being investigated. The ranking order for total phosphate within the grain between the lines also appears to have changed relative to the earlier tests and it therefore appears that growing conditions are important. Micronutrient levels have also been affected as seen in previous tests.

Therefore we need firm data to support a reduction in IP6 before continuing with this material to show reduced phytate. However there would clearly be interest in material that can accumulate more phosphate from its environment.

The 12 best lines were also adapted into a winter wheat background using the Limagrain variety Zebedee and BC1 lines were produced. The seed was multiplied in a polytunnel selfing generation during the summer of 2009 before further characterisation.

4) Analytical methods

Inorganic P assay from single seed (colorimetric assay).

Ref: Chen, Toribara and Warner. Anal Chem. 28:1756

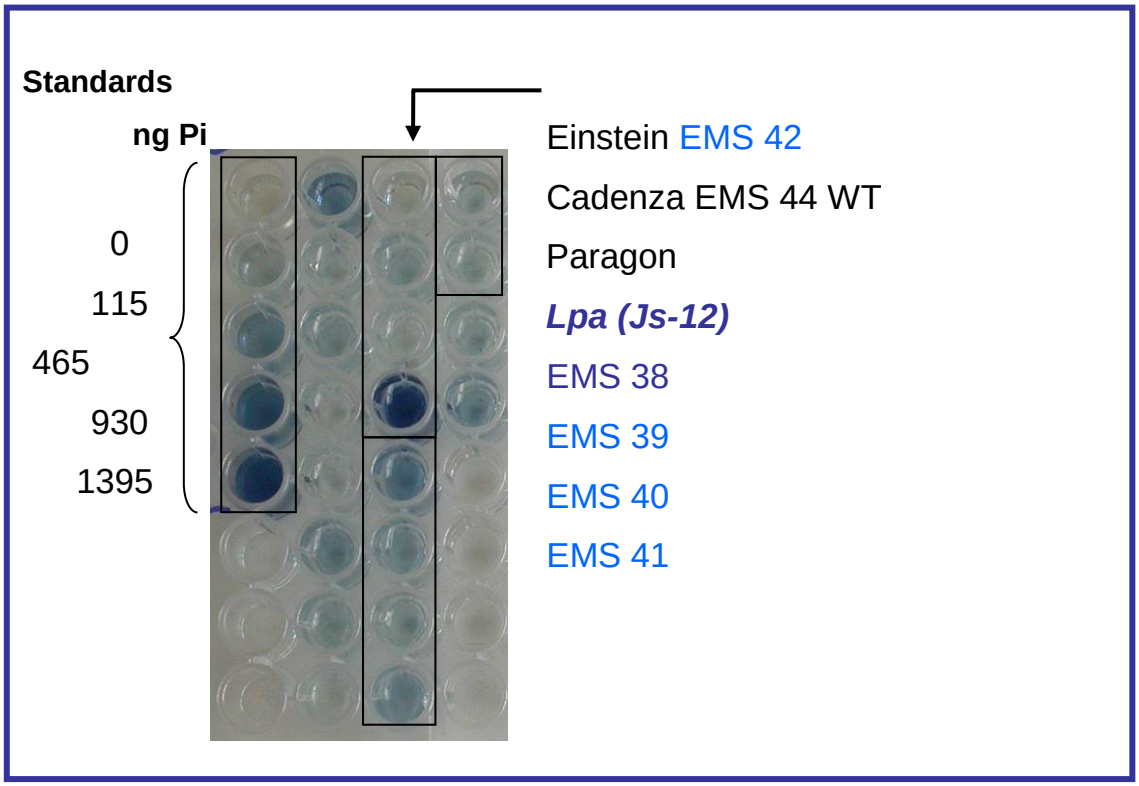


Figure 15. Inorganic P assay from single seed (colorimetric assay for P from Chen *et al*.).

Conclusion

The colorimetric assay is not a 100% reliable technique, which has made selection difficult. Clearly the technique would be unsuitable for a commercial breeding programme which is why the development of markers is important.

Though we have been unable to produce a finished product we have produced well adapted material and some very interesting new target genes. The combination with Cadenza was clearly a fortuitous choice in that it has identified a new unknown mutation. The screening of the Paragon EMS population has produced a selection of candidates for further investigation and the possibility of an increased uptake of phosphate.

3.2.7. Work Package 4. Development of a toolkit for marker-assisted breeding of the HAP trait.

Introduction

The colorimetric assay described in WP3 is a significant amount of labour-intensive work which is difficult to scale up to the sample numbers required for a breeding program whilst maintaining the necessary accuracy required. It is, therefore, of limited value to plant breeders wanting to select the LPW traits.

Using the colorimetric assay for fine mapping the genes responsible for the LPW phenotype has therefore been the objective of WP4. This activity was guided by pre-existing knowledge supplied by the University of Idaho with the aim of producing markers for plant breeders.

Materials and methods

Initially a list of candidate genes in the phytate biosynthetic pathway were compiled from a study of the mutations in the published literature and revised as the project progressed. High priority candidate genes were chosen on their expected mutant phenotype resulting in a decrease in IP6 without the build-up of Ins-P3/4/5 intermediates.

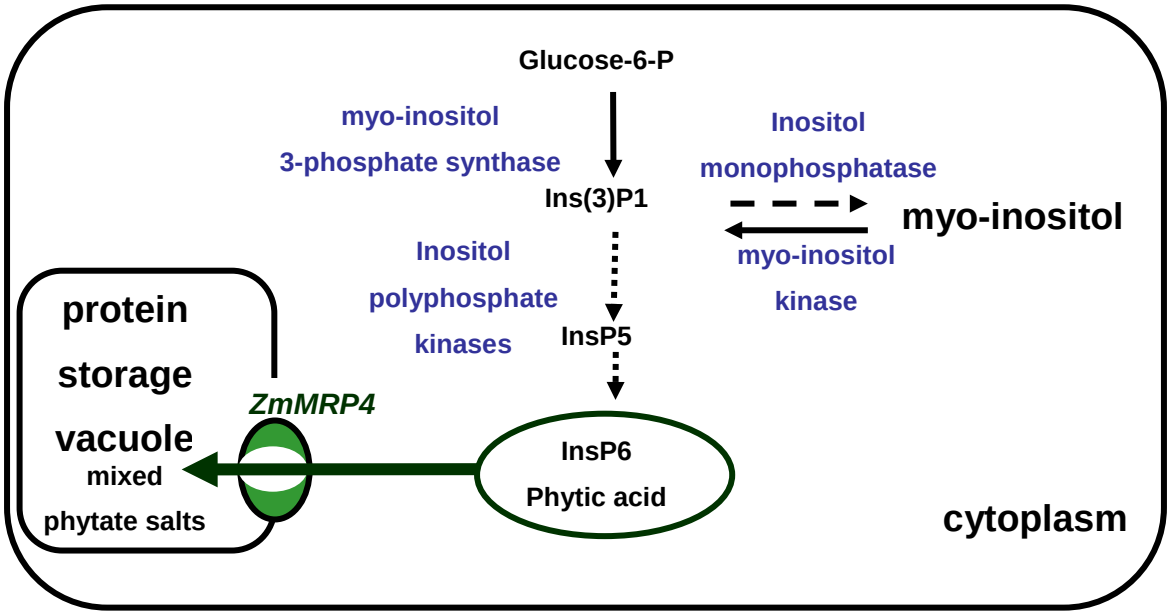


Figure 16. Candidate genes in the phytate biosynthetic pathway.

Phytate pathway mutants:		
	Maize	**Rice**
MIPS	*lpa1-241*	(GM)
MIK	*lpa3*	y
IPK	*lpa2*	
2-PGK		*lpa1-1*
ABC Transporter	*lpa1-241*, (GM)*lpa2-1*	

Figure 17. Phytate pathway mutants

The corresponding genes were identified and sequenced from wheat. Wheat sequences for MIPS and an ABC transporter were initially obtained. Each of the three wheat homeologues for a given gene was characterised and mapped using precise genetic stocks (Nulli-tetrasomic and Kansas Deletion lines). A chromosome location was therefore found which, in conjunction with the double haploid mapping, would rule in or out candidate genes prior to the development of markers.

Sequences from Cadenza and the *lpa* mutant (JS-12) for the MIPS and ABC transporter genes were compared. Whilst homeologues were able to be distinguished for each gene, inter-varietal differences were not generally found. Only one MIPS homeologue was able to be distinguished between the two varieties.

Expression of these genes was also examined in leaf and immature seed tissue. MIPS was found to be abundantly expressed in both tissues, whereas the ABC transporter was only found at relatively low levels in immature seed.

Other biosynthetic genes were not pursued at this point as the rice chromosome locations and rice-wheat synteny suggested that they would not co-locate with the QTLs which had been mapped (section WP3-2).

Gene	Rice	Possible wheat location
IMP	chr 3	4 (5)
2-PGK	chr 2	6
MIK	chr 3	4 (5)
IPK	chr 10, 2, 4	5, 6, 2

Figure 18. Other phytate biosynthesis genes

Several wheat BACs corresponding to the genes which co-locate with the QTLs (MIPS and ABC transporter) have been identified and obtained from the Chinese Spring 6x BAC library held at INRA, Toulouse. Regions of the BACs have been re-sequenced at NIAB to confirm the identity of each homeologue. Three individual BACs have been completely sequenced). This sequence is currently being analysed, and will also be examined for potential SSR polymorphisms which can be used as markers. If none are found, then corresponding flanking regions (or adjacent genes) from Cadenza/Zebedee and the *Lpa* mutant line will be re-sequenced to identify useful polymorphic markers.

Conclusion

Considerable progress has been made and work is currently on-going to achieve the necessary fine mapping to make the markers fully affective tools for a plant breeding programme.

3.2.8. Work Package 5. Determine the effect of P fertilizer treatment on the growth and phosphate metabolism of HAP wheat.

Introduction

Whilst changing the wheat metabolism to affect the availability of phosphate it was considered important to evaluate the effect on other aspects of plant mineral nutrition. This was, therefore, an exploratory work package and was only conducted using the non-adapted US material because access to the UK-adapted material was not possible until late in the programme.

Materials and Methods

Existing specialist field sites were used to study the growth and phosphate metabolism of the natural (spring-sown) low phytate wheat germplasm. The original spring-sown variety from which

the low-phytate germplasm was developed, was used as a control. We conducted two years of field trials at Bunny in 2007 and 2008.

Field experiments completed for 2007 and 2008 on Bunny Farm site, Notts.

Very poor yields due to genetic background (<0.6 t ha^{-1})

No significant variety or P-treatment effect on yield or HI

Analysis of variance

Variate: Yield_at_15%_Moisture_t_ha

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Year stratum	1	0.2184	0.2184	1.51	
Year.*Units* stratum					
P_level	1	0.0152	0.0152	0.11	0.748
Variety	1	0.0241	0.0241	0.17	0.686
P_level.Variety	1	0.0050	0.0050	0.03	0.854
Residual	27	3.9000	0.1444		
Total	31	4.1627			

Variate: Yield_at_15%_Moisture_t_ha

Grand mean 0.560

P_level	high	low
	0.538	0.582

Variety	LOLO	O
	0.532	0.587

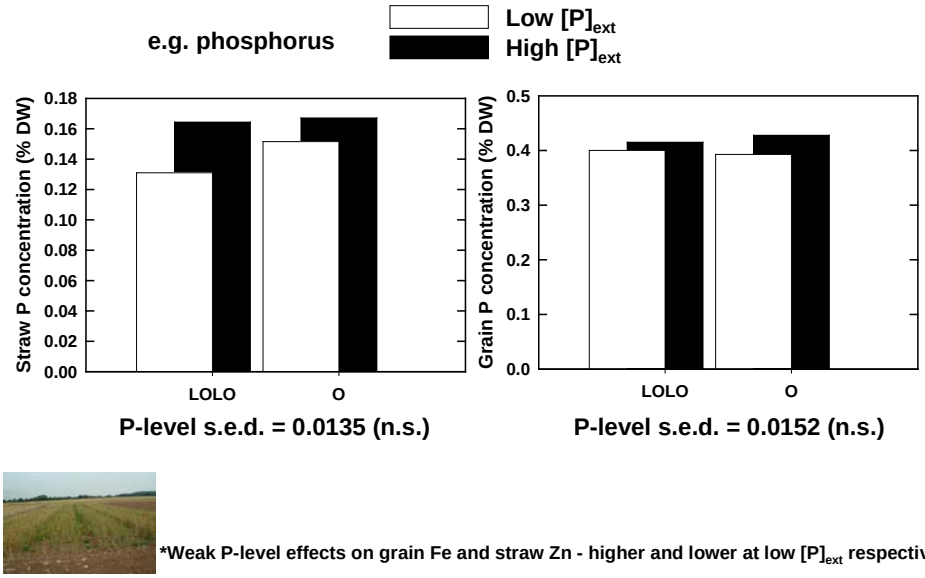
P_level	Variety	LOLO	O
high		0.523	0.553
low		0.542	0.622

Mineral data for 2007 grain and straw analysed, Mineral data for 2008 grain and straw due w/b 8th December 2008

Figure 19, Mineral data for 2007 and 20008 grain and straw from Bunny Farm site, Nottingham.

Field experiments completed for 2007 and 2008, Bunny Farm site

No significant variety or P-level effects on straw or grain mineral composition*



***Weak P-level effects on grain Fe and straw Zn - higher and lower at low [P]$_{ext}$ respectively**

Figure 20. Grain and straw P concentration from field experiments completed in 2007 (left) and 2008 (right) at Bunny Farm site, Nottingham.

Plants were also grown on P gradients under controlled conditions. This was conducted in three experiments and the effect of the LPA on micronutrient content was also studied. It was assumed that a reduction in the phytate content of the plant would affect the availability of the other micronutrients.

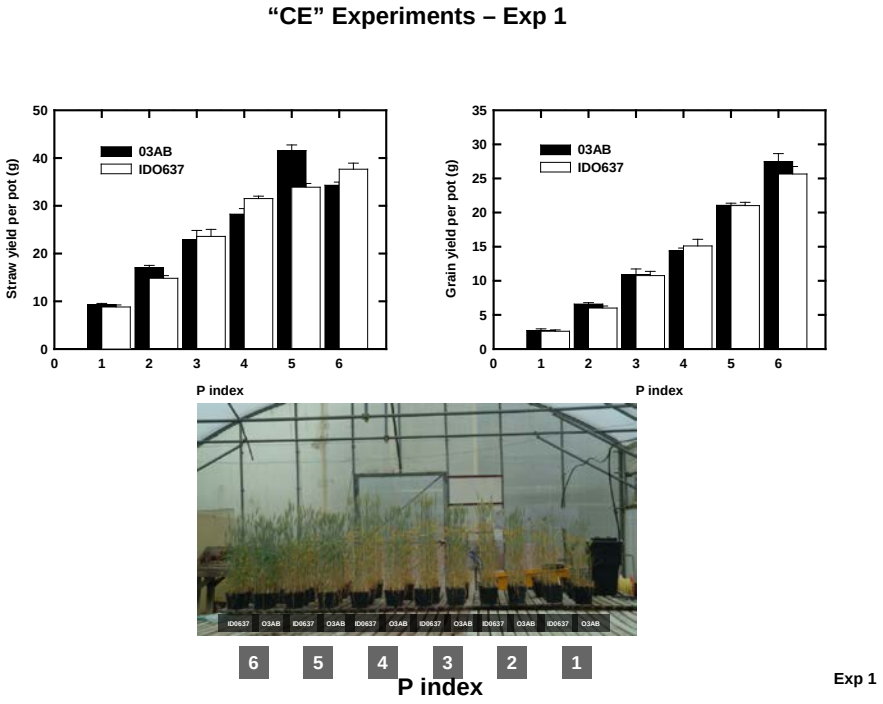


Figure 21. Grain yield for plants grown on P gradients under controlled conditions in 2007 (left) and 2008 (right)

There was no evidence for different response to P in terms of yield of P content however the crop was very low yielding due to the US background. From the three CE experiments, data are inconsistent, but again there was no convincing evidence of genotypic differences in mineral composition between the two lines.

Clearly different soil P levels in the CE experiments had effects on P and other minerals but this was the same in both genotypes.

Conclusion

The important message is that there is no evidence for altered performance or need for altered agronomy with lpa spring wheat (bearing in mind the caveats described above, and ideally this would need testing on UK material). But since phytate is associated with Fe, Zn etc., micronutrient levels should be monitored when the new germplasm is developed, and this was seen in the analysis conducted in WP3-4.

3.3. References

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Appendix A. Trial Diet formulations

		Control	LOLO
Raw Material		%	%
Vit and Micro Min	[]	0.25	0.25
LYSINE HCl	[]	0.5	0.5
THREONINE	[]	0.15	0.15
LIMESTONE FLOUR	[]	0.7	0.7
SALT	[]	0.25	0.25
SODIUM BICARB	[]	0.4	0.4
SOYA OIL	[]	1	1
Ti O2		0. 3	0.3
IDO367 Wheat 72kg/hl	[]	96.75	.
LOLO Wheat 72kg/hl	[]	.	96.75
		100.3	100.3
	Nutrient	Analysis	Analysis
	OILB	3.8975	3.8975
	PROTEIN	15.203425	14.235925
	EECFIBRE	3.50235	2.776725
	ASH	2.78475	2.78475
	SALT	0.556565	0.556565
	CALCIUM	0.439665	0.420315
	PHOS	0.33957	0.349245
	DGP_PIGS_H	0.135641	0.139511
	DGP_PIGS_C	0.169503	0.174341
	SODIUM	0.210538	0.210538
	COPPER	17.9895	17.9895
	DE_ABN	14.0507	14.0507
	DG_LYS	0.727342	0.704703
	DG_MET	0.2054	0.191855
	DG_M+C	0.458885	0.429667
	DG_THR	0.491817	0.469468
	DG_TRY	0.14348	0.13574
	DG_HIS	0.280478	0.261902
	DG_ILEU	0.420669	0.392708
	DG_LEU	0.846659	0.793834
	DG_PH+TY	0.934218	0.872395
	DG_VAL	0.53435	0.498649
	XLYSINE	0.806	0.778039
	XMETH	0.233361	0.217978
	XMET+CYS	0.521483	0.488297
	XTHREON	0.567476	0.540289
	XTRYP	0.163024	0.154219
	HISTIDIN	0.318695	0.297603
	ISOLEU	0.478042	0.446211
	LEUCINE	0.951246	0.891938
	PHE+TYR	1.046738	0.977465
	VALINE	0.621329	0.579823
	C18:2_ABN	1.515624	1.515624

VIT A	7500	7500
VIT D3	1700	1700
VIT E	40	40

3.3.1. Appendix B

Crop Improvement to Reduce Diffuse Phosphorus Pollution

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Abstract

Monogastric livestock contribute significant total phosphorus (P) loads to surface and coastal waters of Europe. Pig and poultry diets are primarily cereal-based, where most grain P occurs as indigestible phytate. To ensure adequate nutrition, the livestock sector uses exogenous inorganic P or phytases in feed rations. Recently, it has become technologically feasible to grow low-phytate varieties of cereals with much greater P availability to monogastrics. These varieties could reduce the need for exogenous P inputs to feed rations and could reduce the P load from agriculture. Here, the impact of low-phytate varieties of cereals in Great Britain (GB) is assessed. A desk-study partitioning the sources of P loads to water was integrated with recent empirical data from studies in which low-phytate cereals were fed to pigs and poultry. Replacing conventional feed by low phytate alternatives would reduce the total P load to GB waters by 0.53% (321 tonnes P y^{-1}), representing 2.73% of the agricultural contribution to the total P load to GB waters. Significant further reductions could be envisaged in the industrial fish farming and human nutrition sectors.

Eutrophication is the enrichment of an ecosystem with nutritive mineral elements causing an in balance within the natural systems. Phosphorus (P) is a major contributor to eutrophication of surface and coastal waters throughout Europe (Haygarth *et al*., 2009; White and Hammond, 2009). To address this problem, the EU aims to reduce P loads to waters through the implementation of the EU Water Framework Directive (WFD, 2000). One of their first objectives was to ascertain the sources of the P entering national waters. Recent estimates for various European countries suggest that agriculture contributes between 10% (Norway) and 80% (Lithuania) of the total P entering their waters (Herbke *et al*., 2005; Withers and Haygarth, 2007; White and Hammond, 2009). Agriculture was estimated to contribute 19.5% of the total P load to the waters of Great Britain (GB; England, Scotland and Wales), with pigs and poultry contributing about 13.6 % of this agricultural P pollution (Table 1; White and Hammond, 2009).

Pigs and poultry are fed cereal-based diets (Nahm, 2002). Typically, 60-80% of the P content of cereal grains occurs as phytate (myo-inositol hexakisphosphate, IP_6; Raboy, 2007, 2009), which monogastric animals, such as pigs and poultry, are unable to digest (Brinch-Pedersen *et al*., 2002). Often farmers must add inorganic P salts to supplement the diets of monogastric animals and provide sufficient P for their adequate nutrition. In addition, farmers often add the enzyme phytase (EC 3.1.3.26) to feed, which degrades phytate to release inorganic phosphate, which can then be utilised by monogastric animals (Baxter et al., 2003; Lei and Porres, 2007). Still, much of the P pollution from pigs and poultry units can originate from undigested phytate. If the phytate content of the feed could be reduced, then P pollution from pigs and poultry units would also be reduced, and the greater P bioavailability in feed would reduce the need for exogenous phosphate and phytase additions to diets of monogastric animals.

Significant natural genetic variation in grain phytate concentrations has been observed among commercial varieties of wheat, maize, barley, triticale, oats, rice, pearl millet and sorghum (reviewed by White and Broadley, 2009). In addition, natural and induced mutants have been described in wheat, maize, barley and rice that have lower seed phytate concentrations, but similar total P concentrations to conventional varieties (Raboy, 2007, 2009; Bohn *et al*., 2008). Genotypes with low seed phytate, and high available phosphate, are now being developed for agriculture.

Dietary studies suggest that pigs and poultry fed cereal grains with low phytate concentrations excrete between 10 and 43% less total P than those fed conventional varieties (e.g. Spencer *et al*., 2000; Veum *et al*., 2001, 2002; Baxter *et al*., 2003; Jang *et al*., 2003; Toor *et al*., 2005; Leytem and Maguire, 2007; Leytem *et al*., 2007). Assuming that feeding pigs and poultry with low phytate cereals reduces total P in their excreta by 20%, it can be calculated that by replacing conventional feed by a low phytate alternative, it would be possible to reduce the total P load to GB waters by 0.53% (321 tonnes P y^{-1}) and the agricultural contribution to the total P load to GB waters by 2.73% (Table 1). These are small, but significant, reductions. If, in addition, total P loads from industrial fish farming, which contributes at least 963 tonnes y^{-1} of total P to GB waters (White and Hammond, 2009), could be reduced by 20%, this would represent a further reduction of 0.32% to the total P load to GB waters.

Human sewage makes a major contribution to the total P load to GB waters (Table 1; White and Hammond, 2009). Much of this is derived from human excreta (Smith *et al*., 2005). Cereals contribute about 31% of the energy input to human diets in GB (Hoare *et al*., 2004). If humans also ate low-phytate cereals, and the P in their excreta was consequently reduced, this could have a large impact on the total P load to GB waters. However, any reduction in the amount of phytate in human diets should proceed with caution because high dietary phytate has been linked to various health benefits (Vucenik and Shamsuddin, 2006).

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Table 1. The potential for reducing total phosphorus (P) loads to GB waters through the use of low-phytate cereals in all pig and poultry rations. Data for total P loads to GB waters assuming current practices were taken from White and Hammond (2009). The total P loads to GB waters if pigs and poultry were fed low-phytate cereals were calculated assuming a 20% reduction in total P excretion.

	Current practice		Feeding low-phytate cereals
	P load (tonnes y^{-1})	% total P load	P load (tonnes y^{-1})
Agriculture	11765	19.51	11444
Pig	751	1.25	601
Poultry	852	1.41	682
Fish farming	963	1.61	
Sewage	28691	47.58	
Total P load	60299		59978