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RESEARCH ARTICLE

Impact of a Novel Phytase Derived from *Aspergillus nidulans* and Expressed in Transgenic *Lemna minor* on the Performance, Mineralization in Bone and Phosphorous Excretion in Laying Hens

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ABSTRACT

The undigested phytates excreted through the feces causes the environmental pollution. The phytase enzyme in feed for layers increases the bioavailability of phosphorus, reduces its excretion and is also reported to be a valuable source of pigment for meat or egg. The objective of this study was to assess the effect of supplementation of phytase expressed intransgenic *Lemna minor* on growth performance, egg quality and mineralization levels of tibia of layers. The experiment was designed with three phytase supplemented groups (B, C and D) and one control group (A) with each 20 replicates of individually caged layers in a completely randomized design. Higher percent increase (P<0.05) in body weights have been recorded in phytase supplemented groups. Results for FCR have shown significant difference (P<0.05) in the phytase supplemented and control group. Increased egg shell thickness has been reported due supplementation of transgenic phytase. An overall significant (P<0.05) increase in egg-production, -weight and the quality of egg-protein has been observed in the layers supplemented with the transgenic phytase. The supplementation of transgenic phytase has significantly (P<0.05) enhanced the deposition of phosphorus and calcium in the bones. An overall significant (P<0.05) decrease in the excretion of phosphorus through feces has been recorded in the treatment groups. The corresponding findings on growth performance, productivity, quality of eggs, bone mineralization and decreased excretion of phosphorus through feces propose phytase derived from *A. nidulans* and expressed in the transgenic *L. minor* as an efficacious and cost effective approach.

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INTRODUCTION

The food bound phytates are difficult to digest by the mono-gastric animals and avian, due to insufficient secretion of phytase (Gao *et al.*, 2013). The undigested phytates cause the environmental pollution, once it is excreted through the feces (Chen *et al.*, 2008). Phytic acid also reacts with amino acids, starch, and other minerals; thereby it reduces their bio availability (Karimi *et al.*, 2013) and raises economic concerns. The phytase enzyme in feed for layers is reported to increase the bioavailability of phosphorus and reduces the phosphorus load on the environment (Selle and Ravindran, 2007; Khan *et al.*, 2010). Microbial phytase (Rutherfurd *et al.*, 2012) and plant phytase (Nyannor *et al.*, 2009; Baber *et al.*, 2012) are currently used as feed supplements.

Peter (1992) reported that layers fed with phytase supplementation had significantly higher egg-production, egg-weights and egg-shell quality than hens fed with the diets without phytase supplementation. Use of *L. minor* (lesser duckweed) as animal feed can be a sustainable alternative (Holshof *et al.*, 2009), due to its high protein content and is used as a source of high-quality protein in poultry. It is also reported to be a valuable source of pigment for meat or egg (Mwale and Gwaze, 2013). *L. minor* meals are used as the supplements in the forage given to ruminants and there is a considerable scope for its use as a source of minerals particularly phosphorus. Therefore, lesser duckweed is economically important as a conventional feed supplement in chicken diets (Haustein *et al.*, 1988). Currently, use of recombinant and transgenic plants having phytase expression is in vogue to improve the bio availability of phosphorus in feed instead of direct supplementation of microbial phytase to animal feed.

Therefore, delivering phytase gene to non-ruminants using transgenic plants are an innovative means to enhance the utilization of phytates bounded phosphorus and minerals uptake (Gontia *et al.*, 2012). This experiment was planned to find out the effects of phytase bound transgenic *L. minor* as a replacement of commercially available recombinant phytase in feed for layers. The objective of this experiment was to assess the effect of supplementation of transgenic phytase on growth performance and egg quality of layers. Secondly, mineralization levels in tibia and amount of phosphorus excreted in the feces were also estimated in layers.

MATERIALS AND METHODS

Layers were handled according to the guidelines of the animal ethics committee of Faculty of Biotechnology, Jeju National University, Jeju-Do, South Korea. 18weeks old healthy layers were procured from the local hatchery.

Housing and feeding regimens: The experiment included three phytase supplemented (B,C and D) and one control group (A) with each 20 replicates of individually caged layers in a completely randomized design (Table 1). The 25±1°C temperature and 18hrs/day photo period were maintained throughout the study.

During the quarantine (two weeks) and experimental period, the layers were offered 120g of feed per day with *ad-libitum* water. Layers in group-A were offered normal feed (Seoul feed, Jeju-Si, S. Korea). The recombinant phytase from *E. coli* for group-B (Biominphytase 5000, Biomin, S. Korea), phytase from transgenic *L. minor* (group-C) and powdered leaves of wild *L. minor* (group-D) were supplemented at specific dose rates (Table 1) for four weeks. The weight gain and feed intake were measured weekly during the experiment. The feed conversion ratio (FCR) was calculated as follows on weekly basis:

$$FCR = F/(W_f - W_0)$$

Where; F=quantity of feed consumed; W_o=Initial body weight; W_f=Final live weight of layers.

Quality analysis of the eggs: The egg-number and -weight were recorded daily. Random samples of eggs from each group were collected twice a week to measure eggshell thickness and albumen height (Haugh unit, HU). Eggshell thickness was measured by using Mitutoyo Micrometer, Japan (Model-CD15CP) at the average of two points at the equatorial region excluding the shell membrane. HU were calculated using the following equation (Eisen *et al.*, 1962):

$$HU = 100 * \log_{10}(h - 1.7w^{0.37} + 7.6)$$

Where;
h=albumen height (millimeters); *w*=egg weight (grams).

Analysis of minerals in tibia and feces: Following the 8h of fasting, layers were re-fed for 2h30min before slaughter to reduce carcass contamination. The layers were sacrificed by cervical dislocation (Rep *et al.*, 2013). The right tibia without soft tissue and diaphysis was stored at -20°C. Later bones were completely dried at 100°C for 12hrs and were grounded for estimation of P, Ca, Mg and Zn. The grounded bones were completely digested with 16M HNO_3 and 30%H_2O_2.

The fresh feces from the each group was weighed. The fecal samples were subjected for mineral analysis to estimate P, Ca, Mg and Na levels. An approximately 250g mixed samples were dried in a forced-air oven (60°C). The concentrations of minerals were measured at specific wavelengths (P-214.914; Ca-317.933; Mg-279.079; Zn-213.856 and Na-589.59nm) by an Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Frentiua *et al.*, 2012).

Statistical analysis: Data were analyzed through one way analysis of variance (ANOVA) and the results were expressed as mean±SD. The means were compared for significance by Tukey's range test at P<0.05 using Statistical Package of the Social Sciences (SPSS).

RESULTS

Effect of phytase supplementation on growth performance: During the current study, a gradual increase in percent body weights have been recorded in all the groups upto 3rd week of the experiment and after that plateau stage for the growth has been observed (Table 2). Although higher percent increase in body weights have been recorded in phytase supplemented groups but there is no significant (P<0.05) difference between the groups supplemented with and without phytase. Moreover, no significant difference (P<0.05) has been observed even among the groups supplemented with recombinant and transgenic phytase. On the contrary two percent increase in body weight, results for FCR have shown significant difference (P<0.05) in the phytase supplemented and control group. An overall decrease in the FCR was observed in all the groups over the period of time. Layers fed on transgenic phytase have shown significantly better FCR than layers from group-B and -D (Table 2).

Effect of transgenic phytase on productivity and quality of eggs: Supplementation with the transgenic phytase has shown significant (P<0.05) increase in egg-production and egg-weight. Layers supplemented with transgenic phytase produced highest eggs (23.0±1.8) followed by layers from group-B (19.0±1.1). Layers from group-C laid heavier eggs (54.2±1.3g) in comparison to

Table 1: Formulation and composition of the experimental feeds including the detail of ingredients used during the preparation of feeds for layer chickens

Ingredients	Experimental diet (%/Kg)			
	A+	B++	C+++	D++++
Maize	65.00	65.00	65.00	65.00
Soybean meal	30.00	30.00	30.00	30.00
Deoiled rice bran	0.60	0.60	0.60	0.60
Salt	0.20	0.20	0.20	0.20
Vitamin premix*	0.10	0.10	0.10	0.10
Trace mineral**	0.10	0.10	0.10	0.10
Calcium carbonate	1.00	1.00	1.00	1.00
Dicalcium phosphate	1.00	1.00	1.00	1.00
DL-methionine	0.14	0.14	0.14	0.14
Lysine	0.10	0.10	0.10	0.10
Sand	0.08	0.08	0.08	0.08
Poultry oil	1.76	1.76	1.76	1.76
Supplements				
rPhytase©	----	1.2g of rPlant/day/ layers	----	----
Phytase t-Plant@	----	----	2g of t-plant/day/ layers	----
WT Plant#	----	----	----	4.4 g of plant/day/ layers
Nutrient composition				
Crude protein (%)	19.5	19.54	19.62	19.62
Crude fat (%)	5.0	5.0	5.0	5.0
Crude fiber (%)	7.0	7.0	7.25	7.4
Calcium (%)	0.7	0.7	0.7	0.7
Total phosphorus (%)	0.9	0.9	1.1	1.07
Non phytate phosphorus (%)	0.3	0.3	0.3	0.3
Methionine (%)	0.5	0.5	0.5	0.5
Lysine (%)	1.15	1.15	1.15	1.15
DCP (%) ¥	16.8	16.8	17.1	17.1
ADE (kcal/kg)$	3,550	3,550	3,550	3,550
Phytase activity(FTU /kg)	0	500	500	0

+A–Control group; ++B–Commercial recombinant phytase; +++C–Transgenic phytase; ++++D–Wild *L. minor;* *Vitamin premix (U/kg diet)= Vitamin A–15,000 IU; D–3,000, IU; E–100 mg; K–4 mg; B1–2 mg; B2–15 mg; B5–25 mg; B6–8 mg; B3–60 mg; B12–0.25 mg; Folic acid–2.5mg; Biotin–0.2 mg, (Bhanja *et al.*, 2005); ©Recombinant phytase; @Transgenic plant with phytase; #Wild type *L. minor* leaves; **Trace mineral (mg/kg diet)= Co–10; I–1; Se–0.2; Fe–50; Mn–70; Zn–100; Cu–10. (Rousseau *et al.*, 2012); ¥DCP–Digestible crude protein; $ADE–Apparent digestible energy

Table 2: Relative increases in body weights and FCR in the treatment groups over the period of study

Group	1st Week		2nd Week		3rd Week		4th Week	
	Per cent growth	FCR	Per cent growth	FCR	Per cent growth	FCR	Per cent growth	FCR
A	2.42±0.06	2.35±0.06	4.91±0.24	2.24±0.15	5.85±0.17	2.02±0.16	6.01±0.65	1.95±0.25
B	3.70±0.24	1.92±0.17	5.72±1.16	1.85±0.29	6.93±1.30	1.76±0.04	7.01±1.37	1.70±0.27
C	4.60±0.18	1.80±0.14	6.80±0.85	1.76±0.09	7.24±0.79	1.69±0.20*	7.35±1.47	1.57±0.16*
D	2.20±0.12	2.00±0.18	4.50±0.17	1.92±0.10	6.31±0.36	1.88±0.17	6.96±0.72	1.76±0.28

*Significantly different from the control group (P<0.05).

Table 3: Comparison of overall mean levels of minerals (%) among the phytase and non- phytase supplemented groups in tibia and feces of laying hens

Parameter/ Mineral	Groups			
	A	B	C	D
Tibia				
P	6.36±0.53a	7.14±0.29a	7.33±0.22b	7.07±0.61a
Ca	13.24±1.10a	16.11±0.28b	14.63±0.62c	12.20±1.19a
Mg	0.25±0.02	0.28±0.02	0.26±0.02	0.27±0.02
Zn	0.79±0.05a	0.62±0.03b	0.62±0.03b	0.61±0.05b
Feces				
P	2.8±0.19a	1.8±0.2a	0.84±0.3b	2.4±0.3a
Ca	5.2±0.29a	4.3±0.21b	3.8±0.44b	4.2±0.37b
Mg	1.9±0.25	1.6±0.20	1.5±0.14	1.8±0.12
Na	2.2±0.20a	1.7±0.43ab	1.7±0.13b	2.4±0.11a

Values (mean±SD) bearing different superscript in a row differ significantly (P<0.05).

group-B (50.1±1.2g). It has been observed that although supplementation of transgenic phytase increased the egg shell thickness in the current study but the differences were not significant (P<0.05) between the treatment groups. Phytase supplementation has shown significant (P<0.05) effect on the quality of egg protein, which was measured by the height of albumin. At the end of the study the highest value of HU (42.5±0.3) was recorded in the layers from group-C followed by group-B (39.6±0.58). No significant difference for HU was observed between the group supplemented with powdered leaves of wild *L. minor* and control group. An overall significant (P<0.05) increase in egg-production, egg-weight and the quality of egg-protein has been observed in the layers supplemented with the transgenic phytase.

Effect of the dietary supplementation of phytase on bone mineralization: The effects of supplementation of transgenic phytase on mineral absorption in bones have been shown in Table 3. The supplementation of transgenic phytase has significantly (P<0.05) enhanced the deposition of P and Ca in the bones. The supplementation with transgenic plant has shown significant increase in P mineralization of bones among the dietary treatments. The calcium mineralization has also shown corresponding increase (14.63±0.62) while maintaining the ratio of 2:1 in the bones. No significant effect of phytase supplementation has been observed on Mg deposition. Even no significant difference among the treatment and control groups has been recorded for Zn deposition in bones.

Effect of phytase supplements on the excretion of phosphorus and other minerals in feces: An overall significant (P<0.05) decrease in the levels of P through feces has been recorded in the treatment groups. An overall 70% and 53% decrease in the excretion of P through feces has been recorded in group–C as compare to group-A and -B, respectively (Table 3). An overall significant (P<0.05) decrease in the excretion of Ca has also been observed in the treatment groups as compared to control group. At the same time no significant (P>0.05) difference was observed between the groups supplemented with phytase from the different sources adopted for the current study. Magnesium and sodium did not show regular patterns, although significant (P<0.05) differences in the sodium levels have been recorded in the treatment groups.

DISCUSSION

It has been observed that the calcium and phosphorus are important minerals which are required for the body growth, egg-production, egg-weights and egg-shell quality (Peter, 1992). These days the production of transgenic plants with phytase expression to improve the bioavailability of phosphorus in feed instead of direct supplementation of animal feed with microbial phytase is in vogue (Gao *et al.*, 2014). It is observed that rDNA fragments or proteins derived from genetically modified (GM) plants have not been detected in tissues, fluids or edible products of farm animals (Shirley and Edwards, 2003). Moreover, the use of GM plants and crops as feed for meat-, egg- and milk-producing animals has fetched the interest of researchers in the current era (Walsh *et al.*, 2012). Therefore, the present investigation focused on the utilization of novel transgenic plant *L. minor* to supplement for phytase through feed for laying hens. It has been reported as a supplementary feed in small holding chicken production (Kabir *et al.*, 2005). *L. minor* absorbs well both phosphorus and nitrogen and is used due to its ability to significantly absorb nutrients and grow exponentially.

To best of author's knowledge and efforts, current study is among the pioneer reports on the use of transgenic *L. minor* as a feed supplement for the laying hens to improve the bioavailability of phosphorus. It is also reported that the supplementation with phytase causes the release of phosphorous from the phytate-mineral complex and can be metabolized by the layers which could reduce the P excretion (Kornegay, 2001). Our finding about the reduction in P excretion by the supplementation of transgenic phytase is in accordance with the earlier findings through the use of transgenic corn (Gao, 2013).

The correlation between the body weight and level of P concentration in feces is an agreement with the earlier studies. Simons *et al.* (1992) suggested that the reduction of P excretion due to phytase supplementation, indicate the bioavailability of P in the body. In the current study, the treatment group supplemented with transgenic phytase has recorded higher percent increase in body weight although no significant (P>0.05) difference has been observed between the groups. Similarly, a linear increase in response to transgenic phytase has been reported for an average daily body weight gain in pigs (Jendza *et al.*, 2005). In our study, significant (P<0.05) increase in egg-production and egg-weight by supplementation with the transgenic phytase is in accordance with the earlier results (Hughes *et al.*, 2008). An overall significant (P<0.05) increase in egg production, egg weight and the quality of egg protein have been recorded in the layers supplemented with the transgenic phytase. Such improvement indicates towards the release and utilization of P by the ingredients of transgenic herb.

The concentration of P and Ca in the bones showed significantly (P<0.05) enhanced levels in group-C with respect to other treatment groups. Our finding that transgenic phytase supplementation has significantly contributed towards the increment of mineral levels in the bones is supported by a finding, stating that a linear increment (P<0.01) in the percentage of bone ash also has been observed (Jendza *et al.*, 2005). Selle and Ravindran (2007) also reported that hydrolysis of the phytate molecule by phytase enzyme results in the release of phosphorus and calcium, which can then be absorbed and utilized. Our results correspond with reports indicating that transgenic plants expressing microbial phytase enhance the utilization of phytate phosphorus and mineral uptake (Xiao *et al.*, 2005; Gontia *et al.*, 2012). The expression of phytase gene in *L. minor* plant is offering the primitive prospects for improving phytate-phosphorus digestibility in laying hen. Phosphorus is also an important component of phospholipids (phosphatidyl choline and phosphatidyl serine) in cell membranes (Crenshaw, 2001).

An overall significant (P<0.05) decrease in the P levels in the group-C with respect to group-A and –B mark towards the significant efficacy of transgenic phytase to reduce the excretion of phosphorus through feces (Table 3). Abioye *et al.* (2010) also reported parallel findings that higher phytase levels are advantageous in reducing the phosphorus concentration of manure. Therefore, the application of this transgenic plant in the poultry feed industry can be a cost-effective option and may be a permissive alternative of recombinant phytase.

Conclusion: The corresponding findings on growth performance, productivity, quality of eggs, bone mineralization and decreased excretion of phosphorus through feces propose phytase derived from *A. nidulans* and expressed in the transgenic *L. minor* as an efficacious and cost effective approach. Further, the reduced excretion levels of P in feces may significantly contribute to fight against the environmental pollution. Our findings would significantly affect the economics of layer farming and would provide a resourceful option for the profitable venture.

Author's contribution: SO, CWC, SJO and DJ conceived the idea and procured the funds.MG, DH, SSS, JHK, RKM, WPP, DJ, HSS and SK carried out the experiment. MG, SSS, NS, NK and DJ wrote the manuscript and authors approved it.

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