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RESEARCH ARTICLE

Effects of Phytase and Citric Acid Supplementation on Scale Mineralization in *Labeo rohita* Juveniles Fed Sunflower Meal Based Diet

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ABSTRACT

Present study was conducted to determine the effect of citric acid (CA), phytase (PHY) and their interaction effects on mineral content in scales of *Labeo* (*L.*) *rohita* juveniles fed sunflower meal (SFM) based diet. Four experimental diets were made containing CA (%) and PHY (FTUkg^{-1}) at 0,0; 2,0; 0,1000 and 2,1000 levels, respectively. Fifteen fish (initial weight 3.15+0.03 g) were allocated to each experimental tank with two replicates for each experimental diet. The duration of the experiment was two months. Water quality parameters i.e. dissolved oxygen, temperature and pH were monitored and kept constant throughout the trial. Results showed that CA supplementation enhanced ($P<0.05$) the mineral content in scales of *L. rohita* juveniles. Similarly, PHY pre-treatment also increased ($P<0.05$) the mineral content in scales of *L. rohita* juveniles. Moreover, both the supplements (CA and PHY) interacted significantly ($P<0.05$) to increase P, K, Cu and Fe contents in scales of *L. rohita* juveniles. In conclusion, CA and PHY pre-treatment of sunflower meal based diet improved mineral deposition in scales of *L. rohita* juveniles. Improved scale mineralization provides more strength to fish to safeguard from infectious diseases and to fight against predation.

INTRODUCTION

Fish feed mostly depends on fishmeal since it provides all necessary nutrients such as vital amino acids, essential fatty acids, vitamins and major minerals, which are important for growth of fish (Zhou et al., 2004). However, rise in demand, fluctuating price and non-consistent supply of the fishmeal arises the need for searching other sources for fishmeal replacement (Gatlin et al., 2007; Lunger et al., 2007; Pham et al., 2008; Lim et al., 2011).

Plant proteins such as soybean meal, canola meal and sunflower meal are considered as encouraging protein sources, which are capable of replacing fishmeal, hence, developing sustainable and environment friendly aquaculture. Feed ingredients obtained from plants are being utilized on a large scale in fish feed industry as they are available throughout the globe and provide cheaper quality proteins as compared to animal sources of protein.

Nevertheless, plant feed is limited in its application in fish feed industry due to certain reasons such as absence of several essential amino acids, less palatability and interference of some anti-nutritional factors, such as phytate, being major problem of all (Libert and Portz, 2005). Phytate, *myo* inositol-1,2,3,4,5,6-hexakisphosphates, is the main storage form of phosphorus in plants (Sugiura et al., 2001), which cannot be utilized by agastric or monogastric fishes which, consequently, suffer deficiency of phosphorus (NRC, 1993). Phytate molecule carries a negative charge and has the capability of binding positively to charged minerals, proteins and starches present in diet, thus, reducing their solubility and absorption (Richard and Thompson, 1997).

Phytase (PHY), an enzyme, has the potential to hydrolyze the phytate present in plant protein sources. Phytase is absent in monogastric and agastric fishes, therefore, they cannot release phosphorus and other phytate bound nutrients. Improved bioavailability of minerals, especially phosphorus, has been reported in fish when fish feeds are supplemented with phytase (Cao et al., 2007).

Supplementation of organic acid, such as citric acid (CA), has been reported to reduce the pH of intestine and acts as chelating agent, which helps in the absorption of cations along the intestine of fish (Ravindran and Kornegay, 1993). Improved body mineralization in beluga has been observed by inclusion of CA in the diet (Khajepour and Hosseini, 2012).

Phytase perform optimally at lower pH (2.5 and 5.0-5.5) compared to normal gut pH (above 6) of agastric fishes. Addition of CA in diet can lower the gastric pH to provide optimum conditions for phytase activity (Simons et al., 1990). Moreover, gastric emptying rate is reduced which provide more time for phytase activity. Hence, it can be hypothesized that addition of CA in the diet may improve the phytase performance in agastric fishes including *Labeo* (*L.*) *rohita* (Sinha et al., 2012). The present experiment was aimed to study the independent and combined effects of PHY and CA on scales mineralization in *L. rohita* juveniles fed sunflower meal based diet.

MATERIALS AND METHODS

The present research work was carried out to study the effects of PHY and CA supplementation on scales mineralization of *L. rohita* juveniles fed sunflower meal (SFM) based diet.

L. rohita juveniles were brought from Government Fish Seed Hatchery, Faisalabad, and acclimatized to experimental conditions for two weeks in large cemented tanks. Prior to start of the experiment, fish were treated with NaCl solution (0.5% w/v) to prevent the infection of ecto-parasites. At the onset of the experiment, fish were transferred to V-shaped tanks (UA system). The volume of V shaped tanks was 70L. Fifteen fish (initial weight 3.15±0.03 g) were allocated to each tank. Two replicates were assigned for each experimental diet. Feed was provided to fish once daily during the trial. Juveniles were fed on basal diet to apparent satiation (Allan and Rowland, 1992). Thermometer, pH meter (Jenway 3510, UK) and dissolved oxygen (DO) meter (Jenway 970, UK) were used to monitor temperature, pH and DO, respectively, during the study period. Other water quality parameters like electrical conductivity and water hardness were also checked throughout the study period. Capillary system was used for the aeration of all the tanks round the clock. The trial was executed for two months.

Feed ingredients and experimental diets

The feed ingredients were purchased from a commercial feed mill. These ingredients were ground, sieved to appropriate size and chemically analyzed prior to the formation of experimental diets (AOAC, 1995). The composition and proximate analysis of experimental diet is shown in Table 1 and 2, respectively.

The present study was carried out to determine the effect of CA, PHY and their interaction on mineral content (mg/g) in scales of *L. rohita* juveniles fed sunflower meal (SFM) based diet. Trail was carried out in 2×2 factorial experiments under Completely Randomized Design. Two levels of phytase (0 FTU and 1000 $FTUkg^{-1}$) and two levels of citric acid (0 and 2%) were used to supplement the diet by pretreatment method resulting in the formulation of four experimental diets namely SFM1, SFM2, SFM3 and SFM4. The SFM1 was pretreated without any supplementation of PHY (0 FTU/kg) and citric acid (0 %); whereas, SFM2 and SFM3 were treated with 2% CA and 1000 FTU/kg PHY, respectively. The SFM4 was supplemented with both the supplements simultaneously (2% CA and 1000 FTU/kg).

Table 1: Composition (%) of the experimental diets

Ingredients	SFM1	SFM2	SFM3	SFM4
Sun flower	65	65	65	65
Wheat flour	15	13	15	13
Rice polish	10	10	10	10
Fish meal	5	5	5	5
Soybean oil	3	3	3	3
Vitamin premix*	1	1	1	1
Mineral mixture**	1	1	1	1
Citric Acid (CA) (%)	0	2	0	2
Phytase (PHY) (FTU)	0	0	1000	1000
Total	100	100	100	100

PHY was added at the expense of wheat flour; *****Each Kg of Vitamin premix contained Vitamin A 15 M.I.U; Vitamin D3 3 M.I.U; Nicotinic acid 25000 mg; Vitamin B1 5000 mg; Vitamin E 6000 IU; Vitamin B2 6000 mg; Vitamin K3 4000 mg; Vitamin B6 4000 mg; Folic acid 750 mg; Vitamin B12 9000 mcg; Vitamin C 15000 mg; Calcium pantothenate 10000 mg; **Each Kg of mineral mixture contained Ca (Calcium) 155 gm; Mn (Manganese) 2000 mg; P (Phosphorous) 135gm; Cu (Copper) 600 mg; Mg (Magnesium) 55gm; Co (Cobalt) 40 mg; Fe (Iron) 1000 mg; I (Iodine) 40 mg; Zn (Zinc) 3000 mg; Se (Selenium) 3 mg; Na (Sodium) 45gm

The process of pre-treatment was as follows: A paste was formed by mixing 1 kg of the ground ingredients with 1.5 L of distilled water. This paste was incubated at 40°C for 15.5 h in order to provide optimum conditions to the enzymes. Later on, it was oven dried at 60°C for 12.5 h to remove the moisture contents. This dried mixture was crushed to obtain powdered form. Vitamin premix and mineral mixture were added to the dried powder. Finally, soybean oil was mixed (Nwanna et al., 2008). Pellets were obtained by using hand pelletizer. Refrigerator was used to store the pelleted diet throughout the feeding period.

Table 2: Proximate analysis of the experimental diets

Diets	SFM1	SFM2	SFM3	SFM4
Dry matter (%)	97.59±0.04	97.51±0.05	97.94±0.20	97.67±0.10
Crude protein (%)	33.93±0.73	34.62±0.84	34.06±0.52	34.44±1.0
Ether extract (%)	10.81±0.41	11.17±0.06	11.11±0.25	11.26±0.33
Gross energy (Kcal/g)	3.88±0.06	3.93±0.03	3.97±0.05	3.86±0.03

Feeding protocol

Prescribed diets were fed to the juveniles of *L. rohita* at the rate of 2% of live wet body weight. After three hours of feeding sessions, the uneaten diet was drained out of each tank by opening the drain valves. After each feeding session the tanks were washed, refilled with water and fish were restocked.

Sample collection

At the completion of trial, clove oil solution (3000 mg/L for 40-60s) was used to anaesthetize the fish and a sharp blow was given on the head to kill them. Mucous of both lateral surfaces was wiped off and scales were removed using scalpel. Distilled water was used to wash the scale samples obtained from each tank. After washing, the scale samples were oven dried for two hours at 110°C. Afterwards, scale samples were defatted with anhydrous ethyl ether for seven hours and crushed in mortar and pestle. Before mineral estimation, samples were dried again and finally weighed.

Chemical Analysis of diet

The samples of feed ingredients and diet were homogenized by using a motor and pestle and were analyzed by following standard methods of AOAC (1995). In order to determine moisture, samples were oven-dried at 105°C for 12 hours. Micro Kjeldahl's apparatus was used to determine crude protein. Ether extraction was carried out using Soxtec HT2 1045 system. The determination of crude fiber was conducted by measuring the loss of dried lipid-free residues, on ignition, after digestion with 1.25% H_2SO_4 and 1.25% NaOH. Samples were ignited in electric furnace (Eyela-TMF 3100) at 650°C for 12 hours in order to estimate crude ash.

Mineral estimation of scales

For mineral estimation, boiling nitric acid and perchloric acid mixture (3:1 ratio) was used to digest the scale samples (AOAC, 1995). After appropriate dilution, Atomic Absorption Spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Japan) was used to estimate minerals, including Ca, Mg, Fe, Cu, Zn and Mn. Analysis of P was conducted through UV Spectro-photometer (Hitachi U-2001) at 720 nm wavelength by using molybdate reagent method. Flame photometer (Jenway PFP-7, UK) was used to estimate Na and K.

Statistical analysis

Two-way analysis of variance was used to analyze the results (Steel et al., 1996). Results were considered significant at $P<0.05$ (Snedecor and Conhran, 1991). P-values of two-way analysis of variance can be used to confirm the responses of two levels of supplements and their interaction. As only two levels of additives were used, therefore, no post hoc test was applied. CoStat computer package (Version 6.303, PMB 320, Monterey CA, 93940 USA) was used for statistical analyses.

RESULTS

Effect of CA, PHY and their interaction on the scale major mineral contents of *L. rohita* are presented in Table 3. Results showed that CA significantly ($P<0.05$) enhanced the P (2.7%), Ca (2.9%), Na (7.7%), Mg (14.4%) and K (3.7%) content in scales of *L. rohita* juveniles. Similarly, PHY supplementation also increased ($P<0.05$) the level of P, Ca, Mg, Na and K by 3.3%, 3.5%, 18.9%, 9.1% and 4.2% in the scales of *L. rohita.* The interaction between CA and PHY was found significant only for P and K in the scales of *L. rohita* juveniles.

The data showing the effect of CA, PHY and their interaction on minor mineral contents of the scales of *L. rohita* juveniles is given in Table 4. Dietary CA supplementation enhanced the Cu (24.7%), Zn (23.3%), Mn (13.1%) and Fe (24.3%) contents in the scales of fish. Similar increase in minor mineral (Mn: 16.1%, Fe: 30.5%, Cu: 15.3% and Zn: 28.9%) of scales was observed by supplementation of PHY. However, PHY supplementation resulted in more increase in scale mineralization (major and minor) as compared to CA. However, unlike other minerals, Cu showed more significant effect with CA supplementation rather than PHY. Furthermore, both the supplements interacted significantly ($P<0.05$) to improve the Fe and Cu contents in scales of *L. rohita* juveniles.

DISCUSSION

The present experiment was executed to study the independent as well as combined effects of PHY and CA on scales mineralization in *L. rohita* juveniles fed sunflower meal based diet.

Approximately, 80% of phosphorus in plant meal based diets is present in the form of phytate. Since monogastric and agastric fishes lack phytase (Pointillart et al., 1987), these are unable to release bound phosphorus from phytate (NRC, 1993). Phytase supplementation enhances the availability of these cations (Liu et al., 2012) as it has the ability to hydrolyze the phytate and these cations become available to agastric fishes (Mitchell et al., 1997).

Table 3: Effect of phytase and citric acid supplementation on major mineral contents in scales of *Labeo rohita* juveniles fed sunflower meal based diet

Phytase (FTU/kg)	Citric acid (%)	Diet	P (%)	Ca (%)	Mg (%)	Na (%)	K (%)
0	0	SFM1	8.06	9.36	0.9	2.2	7.16
	2	SFM2	8.28	9.63	1.03	2.37	7.43
1000	0	SFM3	8.33	9.69	1.07	2.4	7.46
	2	SFM4	8.47	9.94	1.24	2.61	7.5
PSE			0.009	0.011	0.010	0.009	0.011
Analysis of variance	Phytase		.0000***	.0000***	.0000***	.0000***	.0001***
	Citric Acid		.0000***	.0000***	.0001***	.0000***	.0002***
	Phytase× Citric Acid		.014*	.336 ns	.080 ns	.067 ns	.001***

Results were considered significant at P<0.05; Data in the above table are means of three replicates; PSE=pooled SE= √(MSE/n) (where MSE=mean-squared error).

Table 4: Effect of phytase and citric acid supplementation on minor mineral contents in scales of *Labeo rohita* juveniles fed sunflower meal based diet

Phytase (FTU/kg)	Citric acid (%)	Diet	Mn (µg/g)	Fe (µg/g)	Cu (µg/g)	Zn (µg/g)
0	0	SFM1	2.365	47.415	23.635	27.515
	2	SFM2	2.675	58.935	29.48	33.92
1000	0	SFM3	2.745	61.885	27.24	35.48
	2	SFM4	3.055	76.045	35.665	42.555
PSE			0.010	0.351	0.321	0.346
Analysis of variance	Phytase		.0000***	.0000***	.0000***	.0000***
	Citric Acid		.0000***	.0000***	.0001***	.0000***
	Phytase× Citric Acid		1.00 ns	.020*	0.015*	.388 ns

Results were considered significant at P<0.05; Data in the above table are means of three replicates; PSE=pooled SE= √(MSE/n) (where MSE=mean-squared error)

Results of the present experiment showed that phytase supplementation increased mineral contents in scales of *L. rohita* juveniles. Mineral bioavailability might be increased by PHY supplementation as it would hydrolyze the phytate. Hydrolyzation of phytate caused the release of bound mineral which became available for absorption thereby increasing the scale mineralization (Shah et al., 2015). Similarly, improved P contents were also recorded in the scales of *Pagrus major* fed on phytase treated diet (Laining et al., 2012). Similar result was also witnessed in *Oncorhynchus mykiss*, which was fed on phytase added diet resulting in significantly improved P and Ca contents in fish scales. Likewise, Liebert and Portz (2005) also reported improved scale mineralization in Nile tilapia with the addition of dietary microbial phytase. Similarly, phytase treatment resulted in improved bone mineralization in *Salmo salar* (Denstadli et al., 2006) and red sea bream (Laining et al., 2012)

The increased scale mineralization due to CA supplementation, in the present study, might be due to the decrease in gut pH, which may cause the breakdown of mineral compound which formed such chelated mineral complexes that can easily be absorbed. Khajepour and Hosseni (2010) also reported improved mineral deposition in the scute of beluga, *Huso huso*, which was fed 2% CA in a soybean meal based diet. Improved bone P, Ca and Zn contents were observed in rainbow trout fed on diet supplemented with CA (Pandey and Satoh, 2008).

In the current study, a synergism between the supplements (CA and PHY) was also observed to improve the mineral deposition in the scales of *L. rohita* juveniles fed sunflower meal based diet. However, this synergism was significant only for P, K, Fe and Cu. *L. rohita*, being agastric fish, lacks acidic secretion from gut which can provide optimum pH to PHY. Therefore, apart from its individual effect, CA also provides optimum pH (around 5.5) for PHY (Simons et al., 1990). Hence, decrease in pH with CA supplementation might be the reason for the better performance of PHY which resulted in an increased scale mineralization of juveniles (Baruah et al., 2005). Till date, no study has been conducted to analyze the interaction effect of citric acid and phytase for scale mineralization. However, Baruah et al. (2005) observed increased bone mineralization on *L. rohita* fingerlings fed on PHY treated diet added with CA.

In conclusion, the independent effects of CA and PHY as well as their interaction enhanced the scale mineralization of *L. rohita* juveniles fed on sunflower meal based diet. Improved scale mineralization provides more strength to fish to safeguard from infectious diseases and to fight against predation.

Authors' contributions

LS and IL performed the experiment. MA designed the experiment. SZHS and MF analyzed the data and wrote the manuscript. SMH helped in the performance of the experiment.

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