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PHYTASE AND CITRIC ACID SUPPLEMENTATIONS IN SOYBEAN MEAL BASED DIET IMPROVES THE SCALE MINERALIZATION IN *LABEO ROHITA* JUVENILES

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ABSTRACT

Present research work was conducted at Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries. University of Agriculture, Faisalabad Pakistan during the year 2013 to investigate the effect of phytase and citric acid on scale mineralization in Rohu, *Labeo rohita* juveniles fed on soybean meal based diet. Three levels of each of phytase (0, 750 and 1000 FTU/kg) and citric acid (0, 1.5 and 3%) were supplemented in 3^3 factorial arrangement under CRD resulting in the formulation of nine experimental diets. Results indicated improved (P<0.05) contents of P, Ca, Mg, Na, K, Mn, Fe, Cu and Zn in scales of juveniles in response to phytase supplementation. Similarly, improved (P<0.05) deposition of these minerals was also recorded in juveniles having citric acid supplemented diet. However, a significant interaction among both the supplements (phytase and citric acid) was only observed for P and Ca deposition. In conclusion, phytase and citric acid supplementations in soybean meal based diet enhanced the bioavailability of minerals to *L. rohita* juveniles, resulting in improved scales mineralization.

KEYWORDS: Phytase; citric acid; Rohu juveniles; mineralization; *Labeo rohita;* Scales; Pakistan.

INTRODUCTION

Fish meal due to its balanced amino acid and fatty acid profile is most common feed source being used in aquaculture feed industry (3, 4). It is more palatable and digestible to fishes having very less anti-nutritional factors, however, it is expensive and has limited availability (9, 10). Therefore, efforts are being made to replace fishmeal by alternative plant protein sources (7). Among plant sources, soybean meal is considered as the best alternative protein source, because of its availability, high crude protein and low phosphorus contents (26).

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Presence of phytate (an anti-nutritional factor) is the drawback related to the use of plant proteins as feed source. The phosphorus of plant seeds is mainly (60-90%) stored in the form of phytate. Phytate chelates with amino acids, proteins and mineral cations leading to their deficiencies in fish (11). Phytase is an enzyme specific for hydrolysis of phytate (22, 6, 21, 12). This enzyme is present naturally in the gut of many animals, but in monogastric animals like fish its amount is usually very small or even to negligible levels, which causes reduction of dietary phytate digestibility that finally results in reduced availability of amino acids, minerals and proteins from the diet (28).

Supplementation of phytase in feed increased the utilization of phytate phosphorus from diet and its availability in fishes like common carp and rainbow trout (24, 29). Lieberta and Portz (16) reported enhanced nutrients utilization by phytase supplementation to diet. Phytase addition has been proved highly efficient in breaking phytate-protein complex and enhancing protein availability to fish (20). Thus, dietary microbial phytase is considered as vital supplement for makeup of economical and environment friendly aquafeed.

Another approach which is being used in fish nutrition is the supplementation of organic acids. Organic acids when supplemented to feed binds with many positive ions along the whole length of digestive track, ensuring the increased availability of minerals (27, 30). Organic acids inclusion diet lowers the intestinal pH at which phytate becomes physically unstable leading to discharge of phosphorus from its binding and enhancing its availability to fish (31). Khajepour *et al.* (14) reported increased muscle ash, decreased lipids while unaffected moisture and protein contents as a result of citric acid supplementation in the diet of common carp.

Phytase shows best activity at 2.5 and 5.0-5.5 pH ranges (25). Addition of dietary organic acids lowers the intestinal pH which may favour the activity of phytase. Present study was planned to investigate the individual and combined effects of phytase and citric acid supplemented soybean meal based diets on scale mineralization of *Labeo rohita* fingerlings.

MATERIALS AND METHODS

Experimental diet preparation

This study was conducted at Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fishries, University of Agriculture, Faisalabad Pakistan

during the year 2013. Nine experimental diets were formulated by using soybean meal as major protein sources alongwith fish oil as lipid and wheat flour as carbohydrates source (Table 1).

Table 1. Formulation of test diets (percent, as-fed basis)

Phytase (FTU/kg)	**0**			**750**			**1000**		
Citric acid (%)	0	1.5	3	0	1.5	3	0	1.5	3
Ingredients	T1	T2	T3	T4	T5	T6	T7	T8	T9
Soybean meal	56	56	56	56	56	56	56	56	56
Fish meal	12	12	12	12	12	12	12	12	12
Rice polish	12	12	12	12	12	12	12	12	12
Wheat flour	10	10	10	10	10	10	10	10	10
Fish oil	6	6	6	6	6	6	6	6	6
Vitamin premix	1	1	1	1	1	1	1	1	1
Mineral mixture	1	1	1	1	1	1	1	1	1
Ascorbic acid	1	1	1	1	1	1	1	1	1
Chromic oxide	1	1	1	1	1	1	1	1	1

Apart from the supplementation of phytase and citric acid, all experimental diets were similar in nature, containing equal amount of energy (3.67±0.48), nitrogen (27.54±0.49), and lipids (8.00±0.21) level. Three levels of citric acid (0, 1.5 and 3%) and three levels of phytase (0, 750 and 1000 FTU/kg) were supplemented in 3^3 factorial arrangement to formulate nine experimental diets. Feed ingredients were dried and powder ground (0.05 mm) using cereal grinding machine (FFC-45, JIMO, China). All dry ingredients, citric acid and fish oil were mixed electrically. Water was added to make dough which was further processed for pallets making by hand palletizer. Pellets of about 3 mm were made and sprayed with required concentrations of phytase (Phyzme®xP 10000 FTUg-1, Damisco Animal Nutrition. Fin-65 101 Vaasa Finland). Phytase solution was prepared in the way that (2 g powder phytase/ L of water) 1 mL of solution contained 20 FTU in it. Feed pellets were air dried in shade upto 10% moisture and stored in vacuum bags at -20°C throughout the feeding experiment. Three replicates of tanks were used for each experimental diet under completely randomized design. Composition and proximate analysis of diets is given in Table 1 and 2, respectively. Proximate analysis was determined following standard methods of AOAC, 1995 (2). Dried feed samples were oven dried at 105^{o}C until constant weight for dry matter contents; after acid digestion N was determined by micro Kjeldahl apparatus and crude protein by N×6.25 formula. crude fats were assayed through soxtec HT2 1045 system via ether extraction method of Bligh and Dyer (5) and gross energy by using adiabatic oxygen bomb calorimeter (Parr Instrument Co*.,* Moline. USA).

Table 2. Proximate analysis of test diets

Phytase (FTU/kg)	Citric acid (%)	Test Diet	Dry matter (%)	Crude protein (%)	Crude fat (%)	Gross energy (%)
0	0	T1	93.03	28.5	7.57	3.91
	1.5	T2	93.27	28.35	7.43	3.85
	3	T3	93.36	28.75	7.57	3.87
750	0	T4	93.2	28.3	7.51	3.87
	1.5	T5	93.25	28.19	7.54	3.83
	3	T6	93.54	27.9	7.53	3.74
1000	0	T7	93.31	28.58	7.54	3.82
	1.5	T8	93.48	28.75	7.21	3.95
	3	T9	93.67	28.25	7.79	3.83

Experimental fish husbandry

Rohu juveniles were taken from Government Fish Seed Hatchery, Faisalabad, Pakistan. Fish were made ectoparasite free by treating with 5g NaCl/kg at arrival in laboratory and stored at low density in 70L water capacity tanks for 2 weeks with basal diet (T1) feeding. At the beginning of feeding experiment 15 fish were stocked in each of 27 tanks (9 treatments, 3 replicates). Fish were fed at 09:00 and 17:00 hours manually upto apparent satiation 6 days a week. After 2 hours of feeding uneaten diet was siphoned manually. Tanks were supplied with air stone aerators to maintain dissolved oxygen in the range of 5.8-7.3 mg/L. Water temperature and pH were set between 24.9-28.7°C and 7.4-8.6, respectively (optimum conditions for fish culture). Feeding experiment lasted for 12 weeks.

Sampling and analysis

At the end of feeding trial, 5 fish specimens from each tank were starved for 24 hours and dipped in 3000 mg per L clove oil solution for 40-60 seconds to anesthetize (14). They killed by a sharp blow on head. Mucous of both lateral surfaces was wiped off and scales were removed. Scale sample of each tank were pooled, rinsed with distilled water and oven-dried for two hours at 110^{o}C, defatted for seven hours with anhydrous ethyl ether, pulverised in mortar and pestle, dried again, finally weighed and used for minerals estimation. Standard procedures of AOAC (2) were used for minerals estimation after wet digestion. Scale samples were digested in boiling nitric and perchloric acid (2:1) mixture. After required dilutions Na and K were estimated on flame photometer (Jenway PFP-7, UK) while P with the help of

UV-VIS spectrophotometer (U-2001, Hitachi) at 750 nm absorbance after molybdate reagent oxidation. All other minerals (Ca, Mg, Mn, Zn, Cu and Fe) were estimated on atomic absorption spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Japan).

Statistical analysis

Data were analyzed statistically by two-way ANOVA using CoStat computer package (Version 6.303, PMB 320, Monterey, CA, 93940 USA). When significant F values of ANOVA were determined, Tukey's Honestly significant difference test was used to compare individual means. Treatment effects were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Effects of citric acid and phytase supplementations on scale mineralization of L. *rohita* juveniles is given in Table 3. Phytase supplementation showed higher ($p<0.05$) minerals deposition in fish scales. However, supplementation of phytase did not show any dose dependent effect for Ca, Mg, Na, Mn, Fe and Zn deposition in scales. Moreover, both levels of phytase supplementation (750 and 1000 FTU/kg) affected the deposition of P, K and Cu differently. Statistically improved scales mineralization was also recorded by the addition of dietary citric acid in the diet. However, additive effect of both the supplements was significant only for P and Ca deposition in scales.

Most of the P in plant meals is present in the phytate form which is not available to agastric fishes (1, 19). Negative effects of phytate can be alleviated by supplementing phytase in diet (17, 18, 23).

In current experiment, phytase addition caused an improved scale mineralization of Rohu juveniles. Present results favour the previous findings with *Pagrus major* fed phytase treated diet where P level was significantly increased in the scales (15). A similar phenomenon was also observed in *Oncorhynchus mykiss* fed on phytase added diet resulting in significantly ($p<0.05$) improved P and Ca contents in fish scales (8). Liebert and Portz (16) also reported a significant increase in scale mineralization of Nile tilapia by addition of dietary microbial phytase. These results indicated that supplementation of phytase hydrolyzed the phytate leading to improve bioavailability of minerals in fish.

Table 3. Citric acid, phytase and their interaction with scale mineralization of *L. rohita* juveniles fed on soybean meal based diet.

Phytase (FTU/kg)	Citric acid (%)	Test diet	P (%)	Ca (%)	Mg (%)	Na (mg/kg)	K (mg/kg)	Mn (ug/g)	Fe (ug/g)	Cu (ug/g)	Zn (ug/g)
0	0	T1	6.41h	12.33g	0.44d	1.23c	0.16c	27.35d	18.44d	9.65f	88.50g
	1.5	T2	7.62e	15.03e	0.58c	1.28b	0.28a	28.55c	18.61cd	11.00e	94.50f
	3	T3	7.53f	14.97e	0.58c	1.28b	0.22b	28.75c	18.92bc	11.38d	95.65ef
750	0	T4	7.64e	14.58ef	0.56c	1.28b	0.16c	28.60c	18.48d	11.4d	96.95de
	1.5	T5	8.41c	16.47d	0.64b	1.38a	0.23b	29.65a	19.25ab	12.61b	104.30c
	3	T6	8.51c	17.28c	0.68a	1.37a	0.23b	29.25ab	19.06b	12.11c	105.50bc
1000	0	T7	7.24g	14.10f	0.57c	1.31b	0.24b	22.15f	18.53d	11.46d	98.25d
	1.5	T8	8.62b	19.93b	0.68a	1.38a	0.29a	23.20e	18.94bc	12.74b	106.35b
	3	T9	8.75a	21.05a	0.68a	1.39a	0.28a	28.80bc	19.61a	13.13a	108.75a
PSE			0.03	0.33	0.01	0.02	0.01	0.21	0.2	0.13	0.73
Analysis of Variance											
Phytase			p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	N.S	p<0.05	p<0.05
Citric acid			p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05
Phytase × citric acid			p<0.05	p<0.05	N.S	N.S	N.S	N.S	N.S	p<0.05	N.S

Data are means of triplicate values, Values in each row with different superscript are significantly different (p<0.05), PSE= √MSE/r, where, MSE= Mean Standard Error and r = Replicate Number

Minerals contents of juvenile's scales were significantly affected by dietary citric acid addition except P, Mg and K which also showed numerically higher values in 3% citric acid supplemented fish as compared to control. Khajepour and Hosseni, (13) also reported significantly higher P content in scute of beluga, *Huso huso* fed a citric acid acidified soybean meal based diet as compare to control group whereas they also observed a slight increase in Ca content. Moreover, significant interaction between citric acid and phytase was also recorded for scale mineralization. In conclusion, supplementation of phytase and citric acid, independently, and in combination, improves the scale mineralization in Rohu juveniles, fed on soybean meal based diet.

REFERNCES

1. Anon. 1993. Nutrient Requirement of Fish. Nutrient Requirement of Fish. National Research Council (NRC). Academy Press, Washington, DC
2. Anon. 1995. Official Methods of Analysis. (16th Ed.). Association of Official Analaysical chemistry. (AOAC), Inc., Arlington, Virginia, USA.
3. Anon. 2007. Council Regulation (EC) No. 834/2007 of 28 June 2007 on Organic Production and Labeling of Organic Products and Repealing. Regulation (EEC) No. 2092/91. Off. J. Europ. Union, Legislation.189:1-23.
4. Anon. 2009. Commission Regulation (EC) No. 7 10 /2 00 9 of 5 August 2009 Amending Regulation (EC) No. 889/2008 laying down detailed rules for the implementation of Council Regulation (EC) No. 834/2007. Detailed Rules on Organic Aquaculture Animal and Seaweed Production. Off. J. Europ. Union, Legislation. 204:15–34.
5. Bligh, E.G. and W.J. Dyer. 1995. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol.37:911-917.
6. Cain, K.D. and D.L. Garling. 1995. Pretreatment of soybean meal with phytase for salmonid diets to reduce phosphorus concentrations in hatchery effluents. Progres. Fish-Culturist.57:114-119.
7. Cheng, Z.J.J., R.W. Hardy and J.L. Usry. 2003. Plant protein ingredients with lysine supplementation reduce dietary protein level in rainbow trout (*Oncorhynchus mykiss*) diets, and reduce ammonia nitrogen and soluble phosphorus excretion. Aquaculture. 218:553–565

8. Fox, S.W. and S.J. Davies. 2011. Corn-expressed dietry phytase augments vertebral and scale mineral content in juvenile rainbow trout (*Oncorhynchus mykiss*). Aquacult. Nutri.17:840-852.
9. Gatlin,D.M., F.T.P. Barrows, K. Brown, T.G. Dabrowski, R.W. Gaylord, E. Herman, A. Krogdahl, R. Nelson, K. Overturf, M. Rust, W. Sealey, D. Skonberg, E.J. Souza, D. Stone, R. Wilson and E. Wurtele. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquacult. Res. 38:551–579.
10. Gaylord, T.G., F.T. Barrows and S.D. Rawles. 2010. Apparent amino acid availability from feedstuffs in extruded diets for rainbow trout *Oncorhynchus mykiss*. Aquacult. Nutri.16:400–406.
11. Imorou, T.I., D.E. Fiogbe and P. Kestemont.2008. Mineral status of African catfish (*Clarias gariepinus*) Fed Diets Containing Graded levels of Soybean or Cottonseed Meals. Aquaculture. 275:298-305.
12. Jackson, L.S., M.H. Li and E.H. Robinson.1996. Use of microbial phytase in channel catfish *Ictalurus punctatus* diets to improve utilization of phytase phosphorus. J. World Aquacult. Soc. 27:309-313.
13. Khajepour, F. and A. Hosseini. 2010. Mineral status of juvenile beluga (*Huso huso*) fed citric acid supplemented diets. World Appl. Sci. J. 11:682-686.
14. Khajepour, F., S.A. Hosseini and M.R. Imanpour. 2012. Dietary crude protein, citric acid and microbial phytase and their interaction to influence growth performance, muscle proximate composition and hematocrite of common carp, *Cyprinus carpio* L. juveniles. World J. Zool. 7:118-122.
15. Laining, A., M. Ishikawa, S. Koshio, Lideman and S. Yokoyama. 2012. Dietry inorganic phosphorus or microbial phytase supplementation improves growth, nutrient utilization and phosphorus mineralization of juvenile red sea bream, *Pagrus major*, fed soybean- based diets. Aquacult. Nutri. 18:502-511.
16. Lieberta, F. and L. Portz. 2005. Nutrient utilization of Nile tilapia *Oreochromis niloticus* fed plant based low phosphorus diets supplemented with graded levels of different sources of microbial phytase. Aquaculture. 248:111–119.
17. Liu, L.W., J. Su and Y. Luo. 2012b. Effect of partial replacement of dietary monocalcium phosphate with neutral phytase on growth performance and phosphorus digestibility in gibel carp, *Carassius auratus gibelio* (Bloch). Aquacult. Res. 43:1404–1413.
18. Mitchell, D.B., K. Vogel, B.J.Weimann, L. Pasamontes, A.P.G.M. Van Loon. 1997. The phytase subfamily of histidine acid phosphatases:

isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*. Microbiology. 143:245-252..

19. Pointillart, A., A. Fourdin and N. Fontaine. 1987. Importance of cereal phytase activity for phytate phosphorus utilization by growing pigs fed diets containing triticale or corn. J. Nutri. 29:907–912.
20. Ravindran, V., W.L. Bryden and E.T. Kornegay. 1995. Phytates: occurrence, bioavailability and implications in poultry nutrition. Avian Poult. Biol. Rev. 6:125-143.
21. Riche, M. and P.B. Brown.1996. Availability of phosphorus from feedstuffs fed to rainbow trout *Oncorhynchus mykiss*. Aquaculture. 142: 269–282.
22. Rodehutscord, M. and E. Pfeffer. 1995. Effects of supplemental microbial phytase on phosphorus digestibility and utilization in rainbow trout *Oncorhynchus mykiss*. Water Sci. Technol. 31:143–147.
23. Satoh, S., W.E. Poe and R.P. Wilson. 1989. Effect of supplemental phytate and/or tricalcium phosphate on weight gain, feed efficiency and zinc content in vertebrae of channel catfish. Aquaculture. 80:155–161.
24. Schafer, A., W.M. Koppe, K.H. Meyer-Burgdorff and K.D. Gunther.1995. Effects of microbial phytase on the utilization of native phosphorus by carp in a diet based on soybean meal. Water Sci. Technol. 31:149-155.
25. Simons, P.C.M., H.A.J. Versteegh, A.W. Jongbloed, P.A. Kemme, P. Slump, K.D. Bos, W.G.E. Wolters, R.F. Beudeker and G.J. Verschoor. 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. Brit. J. Nutri. 64:525-540.
26. Storebakken, T.,S. Refstie and B. Ruyter. 2000. Soy products as fat and protein sources in fish feeds for intensive aquaculture. Soy in Animal Nutrition. Drackley, J.K (ed.). Fed. Anim. Sci. Soc., Savoy, IL, USA, p. 127-170.
27. Sugiura, S.H., F.M. Dong and R.W. Hardy.1998. Effect of dietary supplements on the availability of minerals in fish meal: preliminary observation. Aquaculture. 160:283-303.
28. Sugiura, S.H., V. Raboy, K.A. Young, F.M. Dong and R.W. Hardy.1999. Availability of phosphorus and trace elements in low phytate varieties of barley and corn for rainbow trout *Oncorhynchus mykiss*. Aquaculture. 170:285–296.
29. Toko, I.I., D.E. Fiogbe and P. Kestemont. 2008. Mineral status of African catfish (*Clarias gariepinus*) fed diets containing graded levels of soybean or cottonseed meals. Aquaculture. 275, 298-305.
30. Vielma, J., S.P. Lall, J. Koskela, F.J. Schoner and P. Mattila.1998. Effects of dietary phytase and cholecalciferol on phosphorus

bioavailability in rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 163:309–323.

31. Vielma, J., K. Ruohonen and S.P. Lall. 1999. Supplemental citric acid and particle size of fish bone-meal influence the availability of minerals in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquacult. Nutri. 5:65-71.

32. Zyla, K., D.R. Ledoux, A. Garcia and Veum. 1995. An *in vitro* procedure for studying enzymatic dephosphorylation of phytate in maize–soybean feeds for Turkey poults. Brit. J. Nutri. 74:3-17.

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Syed Zakir Hussain Shah	**:**	**Performed the experiment and prepared writeup**
Muhammad Afzal	**:**	**Supervised the experiment**
Mahroze Fatima	**:**	**Statistically analysed the data**
Tanveer Ahmed	**:**	**Assisted in data collection**
Syed Makhdoom Hussain	**:**	**Assisted in data collection**
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0	0	T1	6.41h	12.33g	0.44d	1.23c	0.16c	27.35d	18.44d	9.65f	88.50g
	1.5	T2	7.62e	15.03e	0.58c	1.28b	0.28a	28.55c	18.61cd	11.00e	94.50f
	3	T3	7.53f	14.97e	0.58c	1.28b	0.22b	28.75c	18.92bc	11.38d	95.65ef
750	0	T4	7.64e	14.58ef	0.56c	1.28b	0.16c	28.60c	18.48d	11.4d	96.95de
	1.5	T5	8.41c	16.47d	0.64b	1.38a	0.23b	29.65a	19.25ab	12.61b	104.30c
	3	T6	8.51c	17.28c	0.68a	1.37a	0.23b	29.25ab	19.06b	12.11c	105.50bc
1000	0	T7	7.24g	14.10f	0.57c	1.31b	0.24b	22.15f	18.53d	11.46d	98.25d
	1.5	T8	8.62b	19.93b	0.68a	1.38a	0.29a	23.20e	18.94bc	12.74b	106.35b
	3	T9	8.75a	21.05a	0.68a	1.39a	0.28a	28.80bc	19.61a	13.13a	108.75a
PSE			0.03	0.33	0.01	0.02	0.01	0.21	0.2	0.13	0.73
Analysis of Variance											
Phytase			p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	N.S	p<0.05	p<0.05
Citric acid			p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05
Phytase × citric acid			p<0.05	p<0.05	N.S	N.S	N.S	N.S	N.S	p<0.05	N.S

Data are means of triplicate values, Values in each row with different superscript are significantly different (p<0.05), PSE= √MSE/r, where, MSE= Mean Standard Error and r = Replicate Number