



Effects of Phytase and Citric Acid Supplemented Corn Gluten (30%) Meal-Based Diets on the Mineral Digestibility of *Cirrhinus mrigala* Fingerlings

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Abstract

Phytic acid is a major part of oilseed meals which reduces bioavailability of minerals and protein to fish. The present research project was planned to check the effects of citric acid and phytase supplementation in corn gluten (30%) meal-based diets, on mineral digestibility of *Cirrhinus mrigala* fingerlings. The diets were formulated to supply adequate levels of all required nutrients for normal fish growth. Feces were collected twice a day from each tank. Effect of each treatment on the minerals digestibility was calculated using standard formulae. Highest apparent digestibility coefficient % of minerals (Calcium 68%, Phosphorus 77%, Sodium 64%, Potassium 62%, Magnesium 53%, Iron 64%, Copper 68%, Manganese 67% and Zinc 74%) was observed in the fish fed at 5% citric acid and 500 FTUkg⁻¹ phytase in corn gluten (30%) meal-based diet. Results of the current study indicated that addition of 5% citric acid and 500 FTUkg⁻¹ phytase to corn gluten (30%) meal-based diet was most effective among the levels to release the chelated minerals to *Cirrhinus mrigala* from phytate complexes. Hence, the use of citric acid (5%) and phytase (500 FTUkg⁻¹) improves the fish performance when both were used in corn gluten (30%) meal-based diets.

Keywords: Fishmeal, apparent digestibility coefficient, control diet, test diet

Introduction

In the past fish feed was dependent on the use of fishmeal for essential nutrients and growth factors because fishmeal is enriched with essential nutrients and minerals like indispensable fatty acids, amino acids, vitamins and many growth factors (NRC, 1993; Zhou, Tan, Mai, & Liu, 2004). Increasing demand, rising prices and unstable supply of fishmeal had made it compulsory for a search of alternative protein sources for aquaculture (Pham *et al.*, 2008; Lech & Reigh, 2012). Therefore, the attention has been given to the addition of plant meal-based protein sources in diets for fish. Today, the most important challenge to the aquaculture industry is the formulation and production of cost effective fish feed from better quality protein sources (Baruah, Sahu, Pal, & Debnath, 2004).

Plant by-products are the alternative protein sources in fish feeds but unfortunately they have anti-nutritional factors such as phytate or phytic acid (Liu, Luo, Liang, Wang, & Wu, 2013). Phytate has alarming adverse effects on the fish digestive tract resulting in poor growth performance (Baruah *et al.*, 2004). The use of plant meal has been



suggested as an alternative protein source of fishmeal, as it is affordable, easily available and environment friendly because pollutants are low in plant based feed than that of fishmeal (Dalsgaard, Ekman, Pedersen, & Verlhac, 2009). The use of plant meal is still much less (up to 25% of substitution) in part due to the large amount of indigestible carbohydrates. Phytic acid is the main form of storing minerals in plant seeds (Jorquera, Martinez, Maruyama, Marschiner, & Mora, 2008). Approximately 66% of the total plant phosphorus (P) is usually found in the form of phytate which is practically not available for agastric and monogastric fishes (Baruah *et al.*, 2007). Phytate (phytic acid) binds with minerals to decrease their bioavailability in fish (Helland *et al.*, 2006). Phytate forms mineral-phytate complexes leading to reduce mineral bioavailability from the digestive tract (Greiner & Konietzny, 2006). Myo-inositol hexaphosphatephosphohydrolase is an enzyme which belongs to Class 3: Hydrolases and commonly known as phytase. It is produced by microorganisms or sometimes it is present in some plant ingredients. Mono gastric and a-gastric fishes do not produce this enzyme so they cannot hydrolyze the phytate. Supplementation of phytase in fish feeds has been generally resulted to improve the bioavailability and utilization of plant phosphorus by fish (Cao *et al.*, 2007; Hussain *et al.*, 2016).

The organic acids in feed have been shown to make complexes with Calcium (Ca), Phosphorus (P), Magnesium (Mg), Zinc (Zn) etc. which result in improvement in digestibility of these minerals. Addition of citric acid (CA) to diet for *Cyprinus carpio* has been reported to increase the release of P from phytate in vitro (Khajepour & Hosseini, 2012a). There has been considerable research regarding the effect of dietary acidification on mineral utilization in terrestrial animals, yet studies on fish have been very limited. A recent study has shown that the addition of CA and phytase to fish feed improved P and Ca contents in Beluga (*Huso huso*) fed with SBM diets (Khajepour & Hosseini, 2012b; Liu *et al.*, 2013). Combining a low dose of citric acid with phytase supplemented diets significantly increased the positive effects of the enzyme (Phromkunthong, Nuntapong, & Gabaudan, 2010).

Cirrhinus mrigala commonly known as "mori", one of the major carp species cultured in Pakistan, is a bottom feeder and it feeds on vegetable debris and decaying organic matter. The survival and growth rate of *C. mrigala* depends on the optimum water temperature (Gaddowski & Caddel, 1991). *Cirrhinus mrigala* is grown with other species of major carps and Chinese carps and are generally fed with diets formulated and prepared mixing some feed ingredients of plant by-products (Hussain *et al.*, 2010; Hussain, Javed, Javid, Javid, & Hussain, 2011). *Cirrhinus mrigala* is cultured in polyculture system semi-intensively and it has high commercial value. For the better success of carp farming system, the use of cost effective feed has become necessary to control the economic values of fish farm (Abid & Ahmed, 2009).

Corn gluten meal (CGM) was used as ingredient substitute due to its low price and rich nutrient contents (Hu, Ferrell, Lim, & Davis, 2012). Several studies have been published on digestibility of CGM indicating good results for inclusion levels below 40% of the dietary protein for different species of fish (Alliot, Pastoreaud, Pelaez, & Metailler, 1979; Davies, Williamson, Robinson, & Bateson, 1989). Digestibility of CGM is high, generally, with reported values of 95% and 96% for carp and trout respectively (Pongmaneerat & Watanabe, 1991; Morales, Saenz, Márquez, Díaz, & Moyano, 2013).



The objective of the present study was to investigate synergistic effects of citric acid and phytase supplementation on mineral digestibility of *C. mrigala* fingerlings fed with corn gluten (30%) meal-based diet. This study would be also helpful for the development of cost effective and environmentally friendly fish feeds.

Materials and Methods

This study was conducted in the Fish Nutrition Laboratory, Department of Zoology Government College University, Faisalabad. The area lies between latitude 31.4166° North and the longitude 73.0707° East.

Experimental Fish and Systems

Cirrhinus mrigala fingerlings were purchased from Fish Seed Hatchery, Satiana Road Faisalabad. The fingerlings were acclimatized in laboratory under experimental conditions for fifteen days in fish tanks (GCUF system) that were specially designed for the collection of fecal material. During the acclimatization period, the fish were fed twice daily on the basal diet used in subsequent digestibility study (Allan & Rowland, 1992). Water quality parameters particularly temperature, salinity and dissolved oxygen (DO) were monitored. Oxygen was provided twenty four hours to fish throughout the study period (60 days) by capillary system. Sodium chloride (5 g/L) solution was used to the treatment of ecto-parasites (if present) as well as to prevent fungal infection of the *C. mrigala* fingerlings before starting the experiment (Rowland & Ingram, 1991).

Corn gluten (30%) meal was used as test ingredient to formulate the experimental diet. The diet was divided into three groups, each being 3 kg. Citric acid was added at the level of 0 g (0%), 75 g (2.5%) and 150 g (5%) respectively. The experimental diets were divided into nine test diets with graded levels (0, 500 and 1000 FTU kg⁻¹) of phytase. The nine test diets supplemented with citric acid and graded levels of phytase were fed to nine groups of fish stocked in the experimental tanks. Each of the treatment had three replicates with 15 fingerlings (5.62±0.06 g) in each replicate. Total duration of the experiment was 60 days. The experiment was arranged in completely randomized design (CRD).

Experimental feed Formulation and Preparation

The feed ingredients (Table 1) were purchased from local market and were analyzed for chemical compositions following AOAC (1995) standard methods, prior to the formulation of the experimental diets (Table 2). The feed ingredients were finely ground to pass through a 0.5 mm sieve. All ingredients were mixed in an electric mixer for 8 minutes and fish oil was gradually added during mixing of the diet. During mixing of ingredients, 10% water was also added to form suitable dough and the latter was extruded using SYSLG30-IV experimental extruder to produce pellets (3 mm). The above procedure was followed to formulate the nine corn gluten (30%) meal-based test diets. The required inclusion levels (0, 500 and 1000 FTU kg⁻¹) of phytase (Phyzyme® XP 10000 FTU g⁻¹; Danisco Animal Nutrition, Fin-65101 Vaasa, Finland) were prepared in 25 ml distilled water and sprayed on 1 kg of test diets (Robinson, Li, & Manning, 2002). The control diet (0 FTU kg⁻¹) was sprayed with an equal volume of distilled



water to maintain similar moisture contents. All the prepared diets were air dried (under shady place) and stored at 4°C before use.

Feeding Protocol and Sample Collection

The *C. mrigala* fingerlings were fed two times a day (morning 8:00 am and afternoon 2:00 pm). Fingerlings in each tank were bulk weighed every 15th day during experiment to assess the weight gain of *L. rohita* fingerlings. The fish were fed at 5% of their live wet weight and subsequently adjusted on daily basis intake. From each tank the uneaten diet was drained out after a feeding period of two hours. Before the refilling with water, the tanks were washed completely to remove the particles of uneaten diets. Feces were collected from the fecal collection tube of each tank after three hours of feeding session. Care was taken to avoid the breaking of the skinny fecal filaments in order to minimize mineral discharge. Fecal material of each replicated treatment was dried in oven at 60 °C and stored (in plastic bowls with lid) for further chemical analysis. The experiment lasted for 60 days for the collection of approximately 5 g feces of each replicate.

Chemical Analysis of Feed and Feces

The samples of feed ingredients, experimental diets and feces were homogenized by standard methods (AOAC, 1995). Moisture was determined by oven-drying at 105°C for 12h. Diets and feces samples were digested in boiling nitric acid and perchloric acid mixture (2:1) by following standard methods (AOAC, 1995). After appropriate dilution, mineral contents such as calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn) and manganese (Mn) were estimated using Atomic Absorption Spectrophotometer (Hitachi Polarized Atomic Absorption Spectrometer, Z-8200). Calibrated standards for mineral estimation were prepared from commercially available standards (AppliChem® GmbH Ottoweg4, DE-64291 Darmstadt, Germany). The estimation of sodium and potassium was done through flame photometer (Jenway PFP-7, UK). Phosphorus (P) was analyzed calorimetrically (UV/VIS spectrophotometer) using ammonium molybdate as reagent at 720 nm absorbance through standard methods (AOAC, 1995). Chromic oxide contents in the diets and feces were estimated after oxidation with molybdate reagent using a UV-VIS 2001 Spectrophotometer at 370nm absorbance (Divakaran, Leonard, & Lan, 2002).

Calculation of Apparent Digestibility Coefficient (ADC%)

Apparent digestibility coefficients (ADC%) of minerals in test diets were calculated using the standard formula (NRC, 1993).

$$\text{ADC (\%)} = 100 - 100 \times \frac{\text{Percent marker in diet} \times \text{Percent nutrient in feces}}{\text{Percent marker in feces} \times \text{Percent nutrient in diet}}$$



Data Analysis

Two-way ANOVA was used to find out the significant differences among obtained results (Steel, Torrie, & Dickey, 1996). Corn gluten meal-based citric acid and phytase supplemented diets were compared to determine mineral digestibility using LSD (Least Significant Difference) test at 5% level of probability (Snedecor & Cochran, 1990). The CoStat-computer package (Version 6.303, PMB 320, Monterey, CA, 93940 USA) was used for statistical analysis.

Results

Minerals composition in corn gluten (30%) meal-based diets is presented in Table 3, whereas minerals in fish feces are presented in Table 4. It was observed that there was as equal mineral compositions in all test diets. On the other hand digestibility data (Table 5) showed lowest values when *C. mrigala* fingerlings fed with the control diet (0% CA, 0 FTU kg⁻¹), an improvement was found in test diets supplemented with CA and phytase. Highest ADC% of minerals (Ca 68%, P 77%, Na 64%, K 62%, Mg 53%, Fe 64%, Cu 68%, Mn 67% and Zn 74%) was observed in the fish fed with the test diet supplemented with 5% CA and 500 FTU kg⁻¹ followed by (Ca 65%, P 74%, Na 60%, K 59%, Mg 48%, Fe 62%, Cu 56%, Mn 63% and Zn 71%) fish fed at 2.5% CA and 500 FTU kg⁻¹ level based diet. Significantly lower ADC% (39%, 44%, 42%, 41%, 28%, 29%, 39%, 30% and 44%) of minerals (Ca, P, Na, K, Mg, Fe, Cu, Mn and Zn respectively) was observed in fish fed with the control diet followed up (46%, 57%, 48%, 44%, 31%, 39%, 45%, 45% and 52%) in fish fed at 2.5% CA and 0 FTU kg⁻¹ meal-based test diet. So it can be seen that there are highly significant ($p < 0.05$) differences (up to 32.58%) in digestibility of minerals of the control group and that of the treated levels. The treatment of citric acid and phytase was effective to break down the mineral-phytate complexes and thus it increased the availability of minerals to fish. A significant ($p < 0.05$) interaction was also observed between phytase and citric acid on minerals digestibility.

Discussion

In old era fish feed was dependent on the use of fishmeal for essential nutrients and growth factors because fishmeal is enriched with essential nutrients and minerals (NRC, 1993; Zhou *et al.*, 2004). Due to increasing demands with the passage of time, plant by-products are being used as the alternative protein sources in fish feeds but unfortunately they have anti-nutritional factor (Liu *et al.*, 2013). To overcome this problem supplementation of phytase in fish feeds has been generally resulted to improve the bioavailability and utilization of plant minerals by fish (Cao *et al.*, 2007). In the current study, the maximum values in terms of minerals digestibility for *C. mrigala* fingerlings fed on corn gluten (30%) meal-based test diets were at 500 FTU kg⁻¹ with 5% CA supplemented diet followed by 1000 FTU kg⁻¹ with 5% CA and they were significantly ($p < 0.05$) from other test diets used.

Recent studies have shown that the addition of CA and phytase to the fish feed improved P and Ca content in Beluga (*Huso huso*) fed with soybean meal diets (Khajepour, Hosseini, & Imanpour, 2012; Liu *et al.*, 2013). Combining a low dose (0.22%) of citric acid to the phytase supplemented diets significantly increased the activity of the enzyme (Phromkunthong *et al.*, 2010).



Addition of citric acid to fish diet reduces the pH of stomach and enhances the phytase activity to breakdown the phytate complexes (Baruah *et al.*, 2005). In addition to this, the epithelial cell proliferation in the GIT mucosa is also stimulated by citric acid (Sakata, Adachi, Hashida, Sato, & Kojima, 1995) and thus the digestibility of minerals increases. The results of current study proved the synergetic effect of citric acid and phytase because the digestibility of minerals was significantly improved after acidification of phytase supplemented diets. The digestibility values of Ca, P, Na, K, Mg, Cu, Mn, Zn and Fe were highest at 5% citric acid and 500 FTU kg⁻¹ in case of corn gluten (30%) meal-base test diet. The results of the present study are consistent with that of Baruah *et al.* (2007) who found maximum digestibility of major minerals at 3% citric acid and 500 FTU kg⁻¹ phytase level. They also reported a significant interaction between citric acid (3%) and phytase (500 FTU kg⁻¹) on absorption of P, Na, K, Mg, Mn, and Fe in *L. rohita* fingerlings fed on plant meal-based diet. Similarly, Hussain *et al.* (2016) claimed an improvement in mineral digestibility in phytase treated groups with supplementation of citric acid as compared to control group. They found that phytase inclusion at the level of 400 FTUkg⁻¹ and 4% citric acid efficiently increased mineral contents in *L. rohita* fingerlings. Nearly Similar to our results, Shah, Afzal, Shafaat, Hussain, and Zeeshan (2015) concluded that citric acid (3%) and Phytase (500 FTUkg⁻¹) supplemented soybean meal-based diet showed the potential to improve muscle mineralization of *L. rohita* juveniles individually as well as synergistically. The minute variations in the results of the present study and those of the previous ones might be associated with difference in diet composition, fish species and rearing conditions. Contrary to our results non-significant interactions were observed between phytase and citric acid on mineral utilization of juvenile yellow catfish (Zhu, Xuan, Qiliang, Mingming, & Chunfang, 2014). Soybean meal treated with phytase and citric acid had no effects on the calcium and phosphorus of muscle, scute and serum in beluga *Huso huso* (Khajepour & Hosseini, 2010).

Citric Acid enhances the mineral availability from fish and plant meal and mitigates inhibitory effect of some dietary component (e.g. phytate) on mineral availability in plant meal (Khajepour & Hosseini, 2012b). A significant improvement of minerals (Ca, P, Na, K, Mg, Fe, Cu, Mn and Zn) retention in *C. mrigala* fingerlings fed on acidified phytase treated corn gluten (30%) meal-based diet has confirmed the hydrolysis of dietary anti-nutritional factors like phytate according to this study.

In conclusion, the recent study provides evidence that acidification of phytase treated corn gluten (30%) meal-based diet fed to *C. mrigala* fingerlings increases the minerals digestibility and reduces their discharge into the water. It also proves a great interaction between citric acid and phytase regarding to increase mineral digestibility in fish (*Cirrhinus mrigala*) fed on plant meal-based diet. The optimum levels for supplementation in corn gluten (30%) meal-based diet are 5% citric acid and 500 FTU kg⁻¹ phytase.

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Table 1. Ingredient compositions (%) of corn gluten (30%) meal-based diets

Ingredients	Test diet-I	Test diet-II	Test diet-III	Test diet-IV	Test diet-V	Test diet-VI	Test diet-VII	Test diet-VIII	Test diet-IX
Citric acid (%)	0	2.5	5	0	2.5	5	0	2.5	5
Fishmeal	12	12	12	12	12	12	12	12	12
Corn gluten (30)	56	56	56	56	56	56	56	56	56
Rice polish	12	12	12	12	12	12	12	12	12
Wheat flour*	10	7.5	5	10	7.5	5	10	7.5	5
Fish oil	6	6	6	6	6	6	6	6	6
Vitamin premix	1	1	1	1	1	1	1	1	1
Minerals	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
Phytase FTU kg ⁻¹	0	0	0	500	500	500	1000	1000	1000

*Phytase enzyme was used at the expense of Wheat flour

Table 2. Chemical compositions (%) of feed ingredients

Ingredients	Dry matter	Crude Protein	Crude Fat	Crude Fiber	Ash	Gross Energy (kcal/g)	Carbohydrates
Fishmeal	91.63	47.15	7.56	1.09	25.23	2.13	16.84
Wheat flour	92.45	10.57	2.28	2.49	2.24	2.89	79.53
Corn gluten (30%) meal	93.71	29.19	5.36	6.42	12.09	4.59	42.35
Rice polish	94.09	12.34	13.04	12.87	11.07	3.65	47.03

**Table 3.** Percentage of minerals in test diets of *Cirrhinus mrigala* fingerlings fed on citric acid and phytase supplemented corn gluten (30%) meal-based diet

Treatments	CA (%)	Phytase level (FTU kg ⁻¹)	Ca	P	Na	K	Mg	Fe	Cu	Mn	Zn
T ₁		0	0.142	1.986	0.798	1.260	0.078	0.073	0.745	0.075	0.072
T ₂	0	500	0.142	1.989	0.793	1.264	0.078	0.072	0.743	0.080	0.072
T ₃		1000	0.142	1.975	0.788	1.268	0.077	0.072	0.745	0.084	0.070
T ₄		0	0.143	1.976	0.795	1.271	0.079	0.072	0.748	0.086	0.073
T ₅	2.5	500	0.142	1.979	0.773	1.278	0.079	0.074	0.750	0.090	0.071
T ₆		1000	0.143	1.980	0.784	1.278	0.078	0.074	0.751	0.091	0.073
T ₇		0	0.143	1.982	0.798	1.282	0.078	0.071	0.754	0.093	0.072
T ₈	5	500	0.142	1.972	0.776	1.266	0.081	0.072	0.753	0.094	0.075
T ₉		1000	0.143	1.973	0.771	1.270	0.080	0.070	0.754	0.094	0.072
PSE			0.0007	0.0011	0.00069	0.00112	0.00148	0.00174	0.00076	0.00078	0.00092

Data are means of three replicates

PSE = pooled SE = $\sqrt{\text{MSE}/n}$ (where MSE = mean-squared error)

(Ca: Calcium, P: Phosphorus, Na: Sodium, K: Potassium, Mn: Magnesium, Fe: Iron, Cu: Copper, Mg: Manganese, Zn: Zinc)

**Table 4.** Analyzed mineral compositions in feces of *Cirrhinus mrigala* fingerlings fed on citric acid and phytase supplemented corn gluten (30%) meal-based diets

Treatments	CA (%)	Phytase level (FTU kg ⁻¹)	Ca	P	Na	K	Mg	Fe	Cu	Mn	Zn
T ₁		0	0.096 ^a	1.233 ^a	0.515 ^a	0.823 ^a	0.062 ^a	0.058 ^a	0.506 ^a	0.059 ^a	0.045 ^a
T ₂	0	500	0.063 ^e	0.715 ^e	0.391 ^e	0.678 ^e	0.052 ^e	0.035 ^e	0.396 ^e	0.038 ^e	0.029 ^d
T ₃		1000	0.072 ^c	0.815 ^c	0.447 ^c	0.756 ^c	0.057 ^c	0.041 ^c	0.443 ^c	0.047 ^c	0.033 ^c
T ₄		0	0.085 ^b	0.950 ^b	0.462 ^b	0.788 ^b	0.061 ^b	0.049 ^b	0.459 ^b	0.052 ^b	0.039 ^b
T ₅	2.5	500	0.055 ^h	0.564 ^h	0.340 ^h	0.584 ^h	0.045 ^g	0.031 ^g	0.361 ^g	0.036 ^g	0.023 ^e
T ₆		1000	0.061 ^f	0.657 ^f	0.372 ^f	0.646 ^f	0.048 ^f	0.034 ^f	0.384 ^f	0.042 ^f	0.028 ^d
T ₇		0	0.069 ^d	0.789 ^d	0.420 ^d	0.744 ^d	0.056 ^d	0.039 ^d	0.431 ^d	0.049 ^d	0.033 ^c
T ₈	5	500	0.050 ⁱ	0.498 ⁱ	0.302 ⁱ	0.527 ⁱ	0.041 ^h	0.028 ^h	0.342 ^h	0.034 ^h	0.021 ^e
T ₉		1000	0.058 ^g	0.613 ^g	0.357 ^g	0.626 ^g	0.048 ^f	0.031 ^g	0.383 ^f	0.041 ^f	0.025 ^f
PSE			0.0008	0.0031	0.0017	0.0019	0.0008	0.0009	0.0030	0.0007	0.0011

Means within same columns having different superscripts are significantly different at $p < 0.05$

Data are means of three replicates

PSE = pooled SE = $\sqrt{\text{MSE}/n}$ (where MSE = mean-squared error)

(Ca: Calcium, P: Phosphorus, Na: Sodium, K: Potassium, Mn: Magnesium, Fe: Iron, Cu: Copper, Mg: Manganese, Zn: Zinc)



Table 5. Minerals digestibility of *Cirrhinus mrigala* fingerlings fed on citric acid and phytase supplemented corn gluten (30%) meal-based diet. Means within same column having different superscripts are significantly different at $p < 0.05$

Treatments	CA (%)	Phytase level (FTU kg ⁻¹)	Ca	P	Na	K	Mg	Fe	Cu	Mn	Zn
T ₁		0	39.11 ⁱ	44.29 ⁱ	42.11 ⁱ	41.35 ⁱ	28.37 ^h	28.80 ⁱ	39.01 ⁱ	29.68 ⁱ	43.90 ⁱ
T ₂	0	500	59.25 ^e	66.72 ^e	54.33 ^e	50.33 ^e	38.17 ^e	55.00 ^e	50.61 ^e	55.80 ^e	62.55 ^e
T ₃		1000	54.61 ^g	62.95 ^g	49.10 ^g	46.50 ^g	33.86 ^f	48.88 ^g	46.67 ^g	50.34 ^g	57.82 ^g
T ₄		0	46.46 ^h	56.81 ^h	47.77 ^h	44.30 ^h	30.64 ^g	38.90 ^h	44.82 ^h	45.45 ^h	51.71 ^h
T ₅	2.5	500	65.03 ^b	74.12 ^b	60.03 ^b	58.49 ^b	48.09 ^b	61.94 ^b	56.26 ^b	63.34 ^b	71.13 ^b
T ₆		1000	61.22 ^d	69.60 ^d	56.48 ^d	53.67 ^d	42.89 ^d	58.41 ^d	53.15 ^d	57.68 ^d	65.32 ^d
T ₇		0	56.77 ^f	64.23 ^f	52.70 ^f	47.83 ^f	35.77 ^f	50.63 ^f	48.67 ^f	52.18 ^f	59.02 ^f
T ₈	5	500	67.70 ^a	76.87 ^a	64.41 ^a	61.92 ^a	53.50 ^a	64.23 ^a	58.43 ^a	67.02 ^a	73.88 ^a
T ₉		1000	63.47 ^c	72.03 ^c	58.26 ^c	55.63 ^c	45.29 ^c	60.11 ^c	54.19 ^c	60.27 ^c	68.73 ^c
PSE			0.5999	0.3604	0.4851	0.5317	1.1517	1.5416	0.4927	0.9509	1.5311

Data are means of three replicates

PSE = pooled SE = $\sqrt{\text{MSE}/n}$ (where MSE = mean-squared error)

(Ca: Calcium, P: Phosphorus, Na: Sodium, K: Potassium, Mn: Magnesium, Fe: Iron, Cu: Copper, Mg: Manganese, Zn: Zinc)