AGRICULTURE, FOOD & ANALYTICAL BACTERIOLOGY

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Agriculture, Food and Analytical Bacteriology

Effect of Fertilization on Phytase and Acid Phosphatase Activities in Wheat and Barley Cultivated in Bulgaria

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ABSTRACT

Phytase and acid phosphatases in wheat and barley are the major enzymes which catalyze the release of orthophosphate from phosphorylated substrates. Their activities may be influenced by numerous factors including variety differences, growth conditions, and fertilization. The purpose of this study was to evaluate the effect of nitrogen (N), phosphorus (P), and potassium (K) fertilization on phytase and acid phosphatase activities in wheat and barley varieties which are developed and cultivated in Bulgaria. A randomized block design method was applied to a field experiment to study eight treatments which included the application of N, P, K and the combinations of N x P, N x K, P x K and N x P x K. It was established that increased N contents of both wheat and barley grains stimulated phytase activities. The accumulation of P in the grains resulted in decreases of the enzyme activities. Acid phosphatase activities in wheat and barley were less impacted by the applied fertilizers as evidenced by small statistical differences that were established. No specific trend of K-dependent influence on both enzymes was observed. The application of N- and P-containing fertilizers may be used to modulate phytase activities in wheat and barley. If yielding a crop with increased intrinsic phytase activities is needed, utilization of N-rich fertilizer is recommended.

Keywords: wheat, barley, fertilization, phytase, acid phosphatase, animal nutrition

Agric. Food Anal. Bacteriol. 2:103-110, 2012

INTRODUCTION

Phytase and acid phosphatases in wheat and barley are the major enzymes which catalyze the release of orthophosphate from phosphorylated substrates (Viveros *et al.*, 2000; Centeno *et al.*, 2001). Inorganic phosphate is necessary for seed germination and embryo growth (Brinch-Pedersen *et al.*, 2002). The levels of bioavailable phosphate are also important when wheat and barley are used as feed ingredients for monogastric animals (Pointillart *et al.*, 1987). Non-ruminants are incapable of utilizing phytate-bound phosphorus (P) which may reach up to 80% of total P content (Kirby and Nelson, 1988; Reddy *et al.*, 1989; Perney *et al.*, 1993). Due to the importance of P to

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animal health and the uncertainties of P bioavailability in feed ration, farmers and animal producers are inclined to over-supplement P containing sources to create a margin of safety (Sutton *et al.*, 2001). However, in countries with highly regulated animal waste management, P overload is strictly monitored to prevent pollution of soil and groundwater. In addition, microbial phytase supplementation of animal diets, which is meant to reduce the amount of excreted P by making more of it bioavailable to animals, is very often constrained by expenditure associated with production and application of the enzyme (Afinah *et al.*, 2010). To more precisely formulate P levels in animal diets and avoid elevated feed cost and environmental pollution, while achieving optimal animal performance, more information about the capacity of the intrinsic orthophosphate releasing enzymes in feed ingredients is necessary.

By studying phytate-degrading enzyme activities in legume seeds, cereals, and cereal by-products, Steiner *et al.* (2007) and Viveros *et al.* (2000) estimated intermediate phytase activities in cereals which, however, varied over a wide range. Even for one plant species, data for phytase and acid phosphatase activities reported by different authors varied considerably (Viveros *et al.*, 2000, Greiner and Egli, 2003; Steiner *et al.*, 2007; Zarei, 2007). The variations could be explained by differences in the varieties used for the studies, growth conditions, and fertilization (Liu *et al.*, 2006; Steiner *et al.*, 2007; 2008; Kaya *et al.*, 2009). Although information on phytase and acid phosphatase activities in wheat and barley has been published, little is known about their modulation by nitrogen (N), P, and potassium (K) which are major fertilizing agents used in agriculture to improve soil quality and crop yields. The purpose of this study was to evaluate the effect of N, P and K fertilization on respective seed mineral contents as related to phytase and acid phosphatase activities in wheat and barley cultivated in Bulgaria. Although Ca^{2+} and Mg^{2+} were not included in our fertilization experiment, their contents in seeds were also evaluated and discussed as potential determinants of the enzyme activities (Igamnazarov *et al.*, 1998).

MATERIALS AND METHODS

Experimental field design and fertilization

A field experiment was carried out in 2009/10 near village Sadievo (42° 31′ 1.2″ N, 26° 4′ 58.8″ E), region of Stara Zagora, Southern Bulgaria. Bulgarian varieties wheat (Aglika) and barley (Aheloj 2) were grown in soil type "vertisols" ($pH_{(kcl)}$ 5.3) which contained 16.3 mg NH_4-N /kg, 14.0 mg NO_3-N /kg, 2 mg P_2O_5/100g, and 24 mg K_2O /100g before the experiment. A randomized block design method was used to study eight treatments which included the application of N, P, K, and combinations of N x P, N x K, P x K, and N x P x K. The cereals grown in non-fertilized soil were used as a control. Each treatment consisted of four replications which were performed on plots sizing 5 x 5 m (25 m^2). Ammonium nitrate, triple superphosphate and potassium chloride were applied to field to supply N, P_2O_5 and K_2O at the rates of 100 kg/ha, 120 kg/ha, and 80 kg/ha for the wheat, and 80 kg/ha, 120 kg/ha, 80 kg/ha for the barley respectively.

Cereals (wheat and barley) were field collected and air dried. Representative samples (100 g) were taken, ground to pass a 1 mm sieve and stored in sealed containers. All reagents used throughout the experiments were of analytical grade and bought from Sigma (Buchs, Switzerland).

Determination of N, P, K, Ca, and Mg in cereal grains

Seed samples were digested with concentrated HNO_3 and heated in a MarsXpress microwave digestion system (CEM GmbH, Germany) to determine P, K, Ca, and Mg contents. Concentration levels of K, P, Ca, and Mg were established by using an Optical Emission Spectrometry with Inductively Coupled Plasma (ICP OES), Liberty Series II at 769.896 nm, 213.618 nm, 315.887 nm, and 279.079 nm respectively. N was determined by Kjeldahl method (Bremner, 1996).

Enzyme activity measurements

Intrinsic phytase activities in wheat and barley were determined by the extraction procedure of Grainer and Egli (2003) with some modifications. The method is based on the quantification of inorganic phosphate released by phytase from phytate which was used as a substrate. The liberated phosphate was measured according to the ammonium-molybdate method (James, 1999). Briefly, samples (3 g) were extracted for 30 min with 100 ml 0.25 M acetate buffer solution (pH 5) at constant stirring and room temperature (22°C). Solid particles were removed by centrifugation for 15 min at 6000 g (MPW Med. Instruments, Warsaw, Polska) and supernatants were analyzed for phytase and acid phosphatase enzyme activities. For the phytase evaluation, the reaction mixture consisted of 0.9 ml acetate buffer (0.25 M, pH 5), 2 ml 7.5 mM sodium phytate (Sigma-Aldrich P8810), and 0.1 ml test solution was incubated for 30 min at 37°C. The enzyme reaction was stopped by adding a stop solution (2:1:1 v/v/v) consisted of nitric acid (1:2 v/v nitric acid:water), ammonium molybdate (5%), and ammonium metavanadate (0.235%). The yellow complex, formed after the reaction between the liberated inorganic phosphate and the acidic molybdate/vanadate reagent, was measured with a spectrophotometer (Carl Zeiss, Jena, Germany) at 415 nm. Phytase activity was calculated against a standard curve constructed with potassium dihydrogen phosphate and expressed as unit/kg (U/kg) on a dry matter (DM) basis. One phytase unit was defined as the amount of the enzyme which liberates 1 µmol of inorganic phosphorus per minute from 5 mmol of sodium phytate at pH 5 and 37°C.

Acid phosphatase activity was determined as described by Zyla *et al.* (1989). The method is based on the quantification of the p-nitrophenol released from p-nitrophenyl phosphate by the catalytic action of the enzyme. Reaction mixture contained 1 ml 10 mM substrate (p-nitrophenyl phosphate) dissolved in 0.25 M acetate buffer (pH 5) and 0.2 ml extracted enzyme. After incubation for 30 min at 37 °C, the reaction was stopped by the addition of 5 ml 50 mM NaOH. The intensity of yellow color was measured with a spectrophotometer and the enzyme activity was calculated against a standard curve constructed with graded concentrations of p-nitrophenol. One unit acid phosphatase activity was defined as the amount of the enzyme which liberates 1 µmol of p-nitrophenol per minute under above conditions.

Variability, replication, and statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) program (IBM SPSS Stattistics 17, Somers, NY, USA). Presented results are averaged means of at least two independent experiments ± standard deviations. Mean differences and between-subject effect were established by one-way analysis of variance (ANOVA) using the general linear model procedure and Duncan's multiple comparison test. Statistical differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Effect of fertilization on mineral contents of wheat and barley grains

Plant fertilization is one of the most important intensifying factors of wheat and barley crop production. In addition to crop yield, it influences the quality of harvested product which may include mineral and protein contents, chlorophyll and carotenoides, amino acid composition and enzyme activities (Koszanski *et al.*, 1997; Manolov *et al.*, 1999; Černý *et al.*, 2010). N, P and K are macronutrients which are required by plants in relatively large amounts. They are commonly included in agricultural practices as fertilizing agents to improve soil fertility and modulate plant enzyme activities towards desired crop characteristics (Stewart *et al.*, 2004; Kaya *et al.*, 2009; Balabanli *et al.*, 2010).

In our study, the application of N-containing fertilizers in all cases (N, NP, NK and NPK) resulted in increases of N contents of both wheat and barley grains compared to controls (Tables 1 and 2). Av-

Table 1. Effect of fertilization differences on mineral content of wheat grains

Fertilization, kg/ha	N, %	P, %	K, %	Ca, %	Mg, %
control	1.78 ± 0.15^{bc}	0.24 ± 0.08^{b}	0.41 ± 0.03	0.046 ± 0.008^{b}	0.105 ± 0.009
N_{100}	2.14 ± 0.12^{a}	0.21 ± 0.02^{c}	0.39 ± 0.03	0.048 ± 0.004^{b}	0.093 ± 0.006
P_{120}	1.65 ± 0.12^{c}	0.29 ± 0.03^{a}	0.38 ± 0.05	0.043 ± 0.001^{b}	0.97 ± 0.004
K_{80}	1.75 ± 0.17^{bc}	0.24 ± 0.07^{b}	0.35 ± 0.02	0.042 ± 0.009^{b}	0.107 ± 0.002
$N_{100}P_{120}$	2.16 ± 0.20^{a}	0.25 ± 0.02^{b}	0.39 ± 0.04	0.039 ± 0.004^{b}	0.110 ± 0.002
$N_{100}K_{80}$	2.04 ± 0.09^{ab}	0.24 ± 0.09^{b}	0.38 ± 0.02	0.045 ± 0.006^{b}	0.105 ± 0.001
$P_{120}K_{80}$	1.63 ± 0.10^{c}	0.29 ± 0.03^{a}	0.42 ± 0.09	0.083 ± 0.006^{a}	0.104 ± 0.004
$N_{100}P_{120}K_{80}$	2.16 ± 0.11^{a}	0.32 ± 0.04^{a}	0.38 ± 0.04	0.043 ± 0.002^{b}	0.101 ± 0.003

Data represent average means of at least two independent experiments ± standard deviations.
[a-c] Means in a column with different superscripts differ significantly ($p<0.05$). Data for K and Mg were not found significantly different ($p>0.05$).

Table 2. Effect of fertilization differences on mineral content of barley grains

Fertilization, kg/ha	N, %	P, %	K, %	Ca, %	Mg, %
control	1.51 ± 0.13^{b}	0.23 ± 0.10^{cd}	0.32 ± 0.03^{d}	0.049 ± 0.004^{c}	0.107 ± 0.009
N_{80}	1.78 ± 0.15^{a}	0.22 ± 0.03^{cd}	0.39 ± 0.02^{ab}	0.047 ± 0.003^{c}	0.115 ± 0.001
P_{120}	1.44 ± 0.18^{bc}	0.28 ± 0.04^{ab}	0.40 ± 0.02^{a}	0.055 ± 0.002^{c}	0.124 ± 0.001
K_{80}	1.39 ± 0.11^{c}	0.24 ± 0.02^{c}	0.37 ± 0.03^{bc}	0.051 ± 0.002^{c}	0.107 ± 0.003
$N_{80}P_{120}$	1.90 ± 0.26^{a}	0.24 ± 0.09^{c}	0.34 ± 0.04^{bc}	0.039 ± 0.004^{d}	0.109 ± 0.002
$N_{80}K_{80}$	1.91 ± 0.11^{a}	0.20 ± 0.02^{d}	0.35 ± 0.03^{c}	0.045 ± 0.006^{cd}	0.100 ± 0.001
$P_{120}K_{80}$	1.31 ± 0.07^{c}	0.30 ± 0.09^{a}	0.43 ± 0.02^{a}	0.072 ± 0.001^{b}	0.116 ± 0.002
$N_{80}P_{120}K_{80}$	1.63 ± 0.30^{a}	0.26 ± 0.07^{b}	0.39 ± 0.02^{ab}	0.131 ± 0.002^{ab}	0.118 ± 0.006

Data represent average means of at least two independent experiments ± standard deviations.
[a-d] Means in a column with different superscripts differ significantly ($p<0.05$). Data for Mg were not found significantly different ($p>0.05$).

erage increments of 19.38% and 19.53% in grain N concentrations in response to N fertilization were established for wheat and barley respectively. Our data agreed with Bettaieb-Ben Kaab *et al.* (2006) who reported increased concentrations of N in barley grains compared to control in response to all levels of N fertilization which included 40, 80, and 120 kg/ha. Albrizio *et al.* (2010) reported up to 15.8% average increases in N contents of wheat and barley grains as consequence of N fertilization which is close to our findings. Our results were not unexpected because of plant capabilities to accumulate N upon excessive N soil availability (Golik *et al.*, 2005). According to Przulj and Momčilović (2003), N is accumulated in

wheat and barley during vegetation period to provide optimal protein and nucleic acid syntheses and is translocated to kernel during reproductive phase. Positive response of N content of wheat and barley grains to N fertilization was also reported by Koszanski *et al.* (1997), and Małecka and Blecharczyk (2008).

Phosphate-based fertilization exhibited similar trend of increase in P concentrations in both wheat and barley (Tables 1 and 2). Except for the combined fertilization with P and N, the grains, collected from plots enriched with P, PK and NPK, contained significantly higher P concentrations than non-fertilized grains ($p< 0.05$). Our data agreed with Syltie and Dahnke (1983) who also observed increased P-content responses of two hard red spring wheat cultivars to incremented application of a P-rich fertilizer to soil.

Fertilization of wheat with K_{80} did not influenced K contents of grains. No significant differences in K grain concentrations ($p< 0.05$) among all treatments were observed (Table 1). This is probably due to relatively high initial content of K in soil (24 mg K_2O /100g) before starting the experiment. When wheat is grown in soil containing optimal K concentrations, further increase of K availability may not affect K plant content (Slaton *et al.,* 2008). In barley, significant differences among K contents of grains were established but they were ambiguously related to K dose applied to soil. They were rather due to interrelationships of macronutrients where P (P, NP, PK, and NPK) exerted the most stimulating effect on K accumulation in barley grains (Table 2).

Although Ca and Mg ions were not included in our field experiment as fertilizers, they were quantitatively measured because of possible inter-element relationships (Markert, 1993; Li *et al.*, 2010) and potential influence on phytase and acid phosphatase activities. Indeed, Ca contents of both wheat and barley subjected to the experimental fertilization design were found significantly different with PK and NPK contributing the most. None of the fertilizers or their combinations influenced Mg contents of wheat and barley grains.

Effect of fertilization on phytase and acid phosphatase activities in wheat and barley grains

In our study, nitrogen fertilization (N and NK) and the respective high N contents in seeds (Table 1 and 2) stimulated phytase activities in both wheat and barley (Table 3 and 4). Similarly, Kaya *et al.* (2009) established positive effect of N fertilization on phytase activity in chickpea (Akcin 91) as evidenced by the increased activity of the enzyme (4.3 U/g) compared to control (3.8 U/g) in a response to soil fertilization with N at the level of 60 kg N/ha. According to Eastwood and Laidman (1971), the increased phytase levels at higher N availability is mediated by certain nitrogen-containing compounds including glutamine, purine and pyrimidine nucleotides. Although significantly not different from the controls, highest acid phosphatase activities (absolute values) were estimated in wheat (6 529 ± 130 U/kg) and barley (4189 ± 254 U/kg) seeds containing highest N concentrations. Increasing acid phosphatase activities from 5.27 to 7.87 mmol/kg in wheat leaves which corresponded to increasing N levels ranging from 13.1 to 45.4 mg $N\text{-}NO_3$/kg were observed by Koszanski *et al.* (1997).

In contrast to N, the wheat and barley seeds containing highest P concentrations (P, PK, NPK; Table 1 and 2) exhibited the lowest levels of phytase activities (Table 3 and 4). Although the biological function of phytase is to liberate inorganic P to provide sufficient amounts for germinating seeds and growing plants, it looks that excessive P availability may inhibit the enzyme. By studying the control mechanisms of the phytin-phytase system in wheat embryos, Sartirana and Bianchetti (1967) established that the rate of phytin breakdown was controlled *in vivo* by the concentration of inorganic phosphate, through the inhibition of phytase activity. Statistical decrease of acid phosphatase activity caused by enhanced P concentrations was established only for barley fertilized with $P_{120}K_{80}$ (Table 4).

In addition to P concentrations, Ca ions may also contribute to phytase inhibition. In fact, wheat seeds fertilized with $P_{120}K_{80}$ resulted in highest accumulation of Ca (0.083 ± 0.006%) and one of the lowest lev-

Table 3. Effect of fertilization differences on phytase and acid phosphatase activities in wheat

Fertilization, kg/ha	Phytase Activity, U/kg	Acid Phosphatase Activity, U/kg
control	1759 ± 73^{c}	5381 ± 327 ^{ab}
N_{100}	2349 ± 75^{b}	6529 ± 130 ^{a}
P_{120}	1160 ± 93^{f}	5243 ± 292 ^{b}
K_{80}	1691 ± 137^{cd}	5283 ± 322 ^{ab}
$N_{100}P_{120}$	2168 ± 90^{b}	5317 ± 253 ^{ab}
$N_{100}K_{80}$	3455 ± 184^{a}	5331 ± 224 ^{ab}
$P_{120}K_{80}$	1593 ± 112^{cd}	5246 ± 139 ^{b}
$N_{100}P_{120}K_{80}$	1478 ± 34de	6565 ± 311 ^{a}

Data represent average means of at least two independent experiments ± standard deviations

$^{a-f}$ Means in a column with different superscripts differ significantly ($p<0.05$).

Table 4. Effect of fertilization differences on phytase and acid phosphatase activities in barley

Fertilization, kg/ha	Phytase Activity, U/kg	Acid Phosphatase Activity, U/kg
control	2570 ± 125^{c}	4288 ± 283^{a}
N_{80}	3038 ± 216^{ab}	4189 ± 254^{ab}
P_{120}	1410 ± 85^{d}	3664 ± 40^{abc}
K_{80}	3442 ± 316^{a}	3778 ± 106^{abc}
$N_{80}P_{120}$	2536 ± 204^{c}	3722 ± 228^{abc}
$N_{80}K_{80}$	3372 ± 227^{ab}	3583 ± 170^{bc}
$P_{120}K_{80}$	1564 ± 42^{d}	3509 ± 35^{c}
$N_{80}P_{120}K_{80}$	2763 ± 235^{bc}	3849 ± 205^{abc}

Data represent average means of at least two independent experiments ± standard deviations.

$^{a-d}$ Means in a column with different superscripts differ significantly ($p<0.05$).

els phytase- and acid phosphatae activities (Table 3). Similarly, the barley seeds containing 0.072 ± 0.001% Ca (Table 2) exhibited phytase- and acid phosphatae activities significantly lower than the control (Table 4). According to Igamnazarov *et al*. (1998), the influence of Ca ions on phytase activity is dose-dependent. Low Ca concentrations ($1x10^{-5}$ M) did not influence phytase activity in cotton plants. However, higher Ca concentrations ($5x10^{-5}$ M) reduced phytase activity by 12%.

CONCLUSIONS

Overall mineral soil fertilization influenced phytase and acid phosphatase activities in both wheat and barley grains. Acid phosphatase activities were less impacted as evidenced by small statistical differences that were established. While the increased N contents of seeds stimulated phytase activities, the abundance of P negatively controlled the liberation of phytine-bound P caused by phytase. No specific trend of K influence on both enzymes was established. Therefore, the application of N- and P-containing fertilizers may be used to modulate phytase activities in wheat (Aglika) and barley (Aheloj 2). If yielding a crop with increased intrinsic phytase activities is needed, utilization of N-rich fertilizer may be the choice.

ACKNOWLEDGEMENTS

This research was supported by project "Best Management Practices for Sustainable Crop Production in Bulgaria" financed by International Plant Nutrition Institute (IPNI) USA and K+S KALI GmbH Germany.

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