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Article

Effects of low nitrogen and low phosphorus stress on iron, zinc and phytic acid content in two spring bread wheat cultivars

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Abstract: Iron (Fe) and zinc (Zn) deficiency in cereal grains has deleterious effects on the health of millions of people, especially in developing countries. As wheat, as a staple crop, is consumed in large quantities, its micronutrient content is important. Crops in Africa are often grown under low nitrogen (N) and low phosphorous (P) conditions. The aim of this study was to determine the effect of low N and low P stress on Fe and Zn and phytic acid concentration, in two commercial spring wheat cultivars with excellent baking quality. The two cultivars did not differ significantly for the measured characteristics. Across all treatments the average values for Fe varied between 19.60-28.61 mg kg^{-1}, Zn between 17.68-33.79 mg kg^{-1} and phytic acid between 5.03-6.92 mg g^{-1}. Low P stress lead to the highest values of Fe and Zn, and the lowest value for phytic acid. Phytic acid:Fe and phytic acid:Zn ratios were also highly significantly reduced under low P stress conditions. Low N conditions caused significantly increased Zn levels. Despite this, the phytic acid:Fe and phytic acid:Zn ratios were relatively high under all conditions, indicating a low bioavailability of both Fe and Zn in these wheat cultivars.

Keywords: bioavailability, Fe, nitrogen deficiency, phosphorous deficiency, phytic acid, wheat, Zn

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1. Introduction

Because of its role in photosynthesis and transport, plants require nitrogen (N) in large quantities to attain normal growth and development. The total world N use in 2014 was estimated at 108 937 126 tons of which only 4% was used in Africa [1]. N deficiency is one of the major crop production constraints in the world [2]. Statistics indicate that the sub-Saharan region utilizes very low levels of N for grain crop production, at an average of 11 kg ha^{-1} yr^{-1}, despite the 90 to 120 kg ha^{-1} yr^{-1} recommended rates [3].

Phosphorus (P) is the most widely used fertilizer after N [4]. P deficiency affects about 40% of the cultivated land of the world and it causes loss of productivity and quality [5]. As most of the P is stored in the grain, harvesting grain crops leads to a continuous removal of P from the soil. Consequently, P fertilizer application is required to address soil P deficiencies. Both N and P are essential macronutrients required for vegetative and reproductive plant growth [6]. Farmers in the sub-Saharan region often do not have access to fertilizer, leading to poor N and P status of soils. Fertilizer cost is the main reasons why fertilizer use in the region is low [7].

Sub-optimal concentrations of Fe and Zn in wheat grain cause micronutrient deficiencies in humans, especially in regions where cereals are the basis of the diet. The World Health Organization (WHO) reported that 30% of the world population suffers from anemia, especially woman and children [8]. The largest rates of anemia are found in southern Asia, central Africa and western Africa (46%; 47% and 50%, respectively). The availability of micronutrients for human uptake is also limited by phytic acid concentration. Phytic acid is a substance that can form complexes with cations such as Zn^{2+}, forming insoluble phytates, such as Zn-phytate, which influences the bioavailability of Zn in grains [9]. Approximately 70% of the total phosphorous contained in grains is in the form of phytate. It was reported

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[10] that phytate content affects Fe bioavailability more than the total Fe content, although this was contradicted by another study [11].

The aim of this study was to investigate the effect of low N and low P stress and a combination of the two on Fe, Zn and phytic acid content in two commercial South African spring wheat cultivars with excellent baking quality.

2. Material and methods

2.1. Greenhouse trials

Two commercial South-African spring wheat cultivars, PAN3497 and SST806 (the commercial standard for spring wheat baking quality in South Africa), with excellent baking quality were sown in 2 l pots, filled with 2 kg soil. The soil was collected from 1.5 m deep subsoil with very low nutrient content. The pots were placed in the greenhouse in a randomized complete block design with two factors; treatments and cultivars.

Four treatments were applied to the two cultivars, with three replications, 15 pots per replication in 2016, and 20 pots per replication in 2017. Each pot contained three plants. The trials were carried out from June to the end of October 2016 and the same time in 2017. Greenhouse temperatures were set to 18°C night and 22°C day. Low N, low P stress and a combination of the two were induced according to the protocol given in Table 1. These treatments were tested against an optimal control. The treatments were initiated at three-leaf-stage. Before this, plants were irrigated with deionized water. Once a week pots were flushed with deionized water to prevent salt build up. Treatments were applied twice a week (250 ml nutrient solution per pot). The electric conductivity was maintained at 1.50 mS cm^{-2} until tillering, and 1.80 mS cm^{-2} after tillering.

All treatments received the same micronutrient fertilization as follows: 3.45 mg l^{-1} $C_{10}H_{13}FeN_2O_8$, 0.30 mg l^{-1} $MnSO_4$, 0.13 mg l^{-1} $ZnSO_4$, 0.62 mg l^{-1} H_3BO_3, 0.05 mg l^{-1} $CuSO_4$, 0.02 mg l^{-1} Na_2MoO_4. After ripening, the seeds were harvested and milled into whole wheat

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flour with a laboratory mill (IKA A10 Yellowline analysis grinder, Merck Chemicals Pty Ltd) and then put through a 1 mm sieve. These flour samples were used for the determination of Fe, Zn and phytic acid.

2.2. Total iron and zinc analysis

The extraction steps of Fe and Zn were done according to the dry-ashing method [12]. Approximately 1 g of wheat flour was weighed into glazed, high-form porcelain crucibles and ashed in a furnace at 550 °C for 3 h. Crucibles were removed and left to cool, and 1 ml concentrated nitric acid (HNO_3) was then added for digestion. The samples were then placed in a hot sand-bath until the acid was completely evaporated, after which they were returned to the furnace for 1 h at 550°C for further ashing. After cooling, 10 ml 1:2 HNO_3:H_2O was added to the samples for further digestion. The samples were returned to the hot sand-bath until they became warm. The samples were then transferred to 100 ml volumetric flasks and filled to the mark with distilled water. Samples were filtered and mineral concentrations were measured in triplicate using an Atomic Absorption Spectrophotometer (Spectra AA 300).

2.3. Phytic acid determination

Phytic acid concentration was determined by using a rapid colorimetric procedure based on the reaction between ferric acid and sulphosalicylic acid. The method used was based on that of Dragicevis [13] with some modifications. Ground flour samples (0.25 g) were weighed into glass tubes and 10 ml 5% trichloroacetic acid was added, and placed on a shaker for 1 h, vortexed at 10 min intervals. Five ml of the extract was transferred into 15 ml tubes and centrifuged at 12 000 g for 20 min. Supernatant (0.5 ml) was transferred to a clean glass tube and 1.5 ml WADE reagent (0.3% $FeCl_3 + 6H_2O$; 3% 5”-sulphosalicylic acid) was added into tubes. Then, the samples were centrifuged at 12 000 g for 10 min. The absorbance of the supernatant was read at 500 nm with a Helios gamma spectrophotometer (Erlangen, Germany). The pink colour of the WADE reagent is due to the phosphate ester and is

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unavailable to react with sulphosalicyclic acid, resulting in a decrease in pink colour intensity. The phytic acid concentration was calculated [14] where the absorbance of the standards is subtracted from the absorbance of the WADE reagent to give the decrease in absorbance value.

The phytic acid standard solution was made from dodecasodium salt, from rice (Sigma P-8810, MW: 660.04 g mol^{-1}). A series of standard phytic acid solutions were made from the standard stock solution by appropriate dilutions, with the addition of extraction solutions to simulate conditions similar to the ones for the samples. The concentration of phytic acid in this series were as follows: 10, 50, 100, 150, 200, 250, 300, 350 and 400 µmol 100 ml^{-1}.

2.4 Phytic acid:iron and phytic acid:zinc molar ratios

The contents of phytic acid, Fe and Zn were converted into moles by division through their molar mass or atomic weight (phytic acid: 660.04 g mol^{-1}, Fe: 55.85 g mol^{-1}, Zn: 65.4 g mol^{-1}). The molar ratios of phytic acid:Fe and phytic acid:Zn were calculated [15].

2.5. Statistical analysis

Analyses of variance (ANOVA) were done on the data for both genotypes, four treatments and two seasons [16] as a three factor analysis. ANOVA was also done for the two cultivars separately, for the two seasons combined, in order to determine the effects of treatments on the measured parameters within each cultivar. Differences were tested at a $p<0.05$ level of significance.

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3. Results

The effect of the cultivar itself was not significant for the measured characteristics. The effect of the treatment was highly significant for Fe, Zn and phytic acid, while the season significantly affected Fe and phytic acid concentrations. There was an interaction between the cultivar and the treatment, and cultivar and the season for Zn, but not for Fe and phytic acid concentrations. The interactions between treatments and seasons were highly significant for phytic acid. There were no significant interactions between the cultivars, treatments and seasons (Table 2).

The treatments had large effects on the measured concentrations of Fe, Zn and phytic acid in both the cultivars (Table 3). Across all the treatments the average values for Fe varied between 19.60 mg kg^{-1} and 28.61 mg kg^{-1}. Zn values varied between 17.68 mg kg^{-1} and 33.79 mg kg^{-1} and phytic acid concentrations varied between 5.03 mg g^{-1} and 6.92 mg g^{-1} (Table 4).

Fe concentration was significantly reduced under low N conditions but was similar under low P and a combination of low N and P treatments. Zn increased under low N, low P (a 47.68% increase) and low N and P combined. Phytic acid was significantly reduced under all three treatments, but the reduction was by far the highest (27.31%) under low P conditions (Table 4). The molar ratio of phytic acid:Fe in wheat was significantly increased under low N conditions, but largely decreased under low P (32%) and a combination of low N and P (19.93%). The phytic acid:Zn molar ratio decreased under all three conditions but the effect was by far the highest under low P conditions with a 60.79% reduction (Table 4). There were no significant correlations between Fe and Zn for any of the treatments in the current study (data not shown).

4. Discussion

Several studies have shown wide variation for Fe and Zn concentrations in wheat [17-19]. The Fe and Zn concentrations are determined by genetic and environmental factors [20]. In this study the effect of cultivar on the measured characteristics was negligible, but treatment

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influences were highly significant for both Fe and Zn and phytic acid. Fe concentration varied between 19.6 to 28.61 mg kg^{-1}, while Zn concentration varied between 17.68 to 33.79 mg kg^{-1}. These values were similar to what was reported previously. The average Fe concentration was reported [21] to be between 30 to 73 mg kg^{-1}. Based on a number of studies, the range of Zn concentration was reported to be between 20.4 to 30.5 mg kg^{-1} in wheat grains, with an average of 27.3 mg kg^{-1} [22]. The optimal Zn concentration for human consumption is around 40-60 mg kg^{-1} [23]. Significant correlations between N fertilization and Fe and Zn concentration in wheat grains was previously reported [23], which was not the case in this study.

The highest Fe and Zn, and the lowest phytic acid concentrations were evident under low P stress (with optimal N supply, Table 1) in the present study. This indicated that low P stress was actually conducive to high Fe and Zn content and its bioavailability, as measured by low phytic content, but, although not measured in this study, low P would certainly reduce yield. The highly reduced phytic acid concentration under low P stress was probably due to the fact that 70% of phosphorous in the plant is in the form of phytate [10], meaning that a reduced availability of P would lead to reduced phytic acid. It was reported [24] that the bioavailability of Fe and Zn in staple food crop seeds and grains is as low as 5% and 25%, respectively. Phytic acid reduces the bioavailability of micronutrients [25]. Phytate is a chelating agent, which reduces the bioavailability of divalent cations such as Zn^{2+} and Fe^{2+} [26].

The variations for phytic acid ranged from 3.05 to 6.92 mg g^{-1} in this study, with significant reductions under all three stress treatments. This indicates that bioavailability should increase under low N, P and combined stress conditions. This view was supported by an oats study [27] where it was found that the phytic acid content depends on fertilization management because N and P fertilizer applications increased phytic acid concentrations. Low P stress had a much larger effect on phytic acid than did N and, N and P stress combined.

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The inhibitory effect of phytate on Fe and Zn absorption can further be examined by the molar ratio of phytate to Zn. The mineral bioavailability is higher when the molar ratio is low. There was very large variation for phytic acid:Zn (14.93-38.08) and phytic acid:Fe (15.15-27.77) ratios between the different treatments. It was reported [28] that 55% of Zn was absorbed when phytic acid:Zn ratio was less than 5, while 35% of Zn is absorbed when the ratios were 5-15, and only 15% was absorbed when the ratio was higher than 15. In the current study the phytic acid:Zn ratio was lower under all the treatments compared to the control, but all the values were relatively high (more than 20 for all excluding the low P treatment), indicating an absorption of less than 15%. This is also valid for Fe absorption where the rations were higher than 20 for the control and the low N treatment, indicating relatively poor bioavailability of the Fe [15].

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Table 1. Fertilizer applied (mg l^{-1}) over two years to two wheat cultivars in four treatments in a greenhouse experiment

Chemicals	Optimal		Low N		Low P		Low N and P	
	BT	AT	BT	AT	BT	AT	BT	AT
KNO_3	261	313	0	0	228	273	0	0
K_2SO_4	210	252	210	252	196	235	196	235
KCl	0	0	193	231	56	67	223	268
$NH_4H_2PO_4$	87	104	87	104	0	0	0	0
$Ca(NO_3)_2$	758	909	0	0	797	956	0	0
$CaCl_2$	0	0	353	424	0	0	446	446
$MgSO_4$	348	418	348	418	369	443	443	443

BT = before tillering, AT = after tillering

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Table 2. Analysis of variance for Fe, Zn and phytic acid concentration in two wheat cultivars with four treatments over two seasons

	Cultivar (C)	Treatment (T)	Season (S)	CxT	CxS	TxS	CxTxS
Fe	11.15	197.44**	382.45**	27.39	55.19	6.38	14.86
Zn	0.26	597.08**	0.01	45.27**	173.09**	96.61	0.05
Phytic acid	0.21	10.26**	5.35**	0.24	0.24	2.92**	0.06

**$P \leq 0.01$

Table 3. Average values for measured characteristics in two cultivars with four treatments over two seasons

	PAN3497	SST806	LSD (0.05)
Fe (mg kg^{-1})	24.86	25.82	1.85
Zn (mg kg^{-1})	23.58	23.70	1.09
Phytic acid (mg kg^{-1})	5.89	6.02	0.18
Phytic acid:Fe	20.26	21.12	
Phytic acid:Zn	23.36	26.06	

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Table 4. Average values for two cultivars and two seasons for measured characteristics for four treatments

	Control	Low N	Low P	Low N and P	LSD (0.05)
Fe (mg kg^{-1})	26.25	19.60	28.61	26.90	2.61
Zn (mg kg^{-1})	17.68	20.54	33.79	22.56	1.54
Phytic acid (mg kg^{-1})	6.92	6.56	5.03	5.31	0.25
Phytic acid:Fe	22.28	27.77	15.15	17.84	
Phytic acid:Zn	38.08	26.62	14.93	23.02	