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Research Article

Improving Grain Iron Bioavailability in Bread Wheat Through Integrated Nitrogen and Iron Fertilization

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ABSTRACT

Low nutritional quality of staple foods, including wheat, is the main culprit of iron deficiency in more than half of the world's population. Modern breeding techniques have improved the yield potential of staple food crops, especially wheat, but the nutritional enhancement was overlooked and resulted in cultivars deficient in iron and higher in phytates. The remedy for this problem is iron bio-fortification of high-yielding cultivars. To achieve this target, forty-four genotypes, including varieties and hybrids, were grown in a randomized complete block design (RCBD) at the field area of University of Agriculture, Faisalabad. A combination of nitrogen (50, 100, and 150 kg ha^{-1}) and iron (2% and 4%) fertilizers was tested to identify the best fertilizer combination for improving grain iron contents. Nitrogen was applied in two splits, while iron was foliar applied at milking stage. Higher nitrogen and iron doses increased grain iron contents considerably up to 89.1 µg g^{-1}. But the bioavailability of grain iron was highest (PA/Fe=0.11) in higher nitrogen and low iron levels due to the lowest phytate contents (0.16 mg g^{-1}) in this combination. While moderate nitrogen and low iron increased grain yield (29.9g plant^{-1}) more than any other combination of these two fertilizers. The highest nitrogen dose coupled with low iron concentration proved best for improving grain iron contents and their availability (0.11) without harming grain yield (19 g plant^{-1}). Some genotypes, including C6 and C14, performed better than all other accessions and can be further evaluated for any genetic potential to improve grain iron and its bioavailability. The study concluded that grain iron and its availability for human digestion can be improved not only by conventional breeding efforts and biotechnology but also ergonomically by manipulating the fertilizer doses.

Keywords: Bioavailable iron, Biofortification, Bread wheat, Grain iron, Iron biofortification, Nitrogen application, Phytate

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INTRODUCTION

During nineteen century, green revolution changed agriculture, enabling it to provide steady food supply for increasing world population. This revolution was only possible due to genetic improvement of cereal crops which were resistant to lodging and were more responsive to the fertilizers applied. All this advancement has almost tripled food production to eliminate famine. Despite all this advancement human population still faces hunger. About 828 million people suffered hunger in 2021, which is 46 million more than 2020 and 150 million more compared to 2019 (FAO, 2022). However, this hunger is not massive compared to famine that humans faced in early ages but still is more damaging due to nutrient deficiency.

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The nutrition deficiency had led to hidden hunger. Iron deficiency contributes a major part of this hidden hunger affecting more than 1.2 billion humans around the globe. Children under the age of five are most affected (39.8%) by iron deficiency followed by women especially 36.5% pregnant women are anemic due to iron deficiency around the world. Iron deficiency not only causes anemia but is also responsible for poor motor and cognitive development, maternal mortality, preterm deliveries and low birth weight. The iron deficiency during first five years of newborn haunts his entire life via poor brain structure, function and overall development. If anemia persist one year untreated, the chances of mortality increases as high as 50% (Chami *et al.*, 2019).

Iron deficiency is mostly treated by supplementation followed by food fortification, nutritional modification and bio-fortification. Supplement and food fortification are costly remedies, and their availability is limited mostly in rural areas. To overcome this hindrance bio-fortification and food diversification are promoted to improve iron status in rural populations. Heme are the prime iron sources and sometimes have limited availability to the poor rural masses and thus making food diversification difficult for rural population. In this scenario bio-fortification stands as the one reliable also affordable permanent solution to the iron deficiency problem in less privileged areas (Liberal *et al.*, 2020).

Bio-fortification of wheat can be one in various ways including breeding highly nutritive cultivars or biotechnological genetic modification as done in golden rice and other crops. These strategies however take decades to prove something positive. Agronomic bio-fortification on the other hand provides a quick and efficient way to improve iron profiles in already established cultivars. Soil fertility management by using NPK and organic fertilizers along with micronutrient fertilizers at different growth stages offer best results in this regard (Ramzani *et al.*, 2016).

Iron is not only important for humans buts also critical for plant growth and development. Iron becomes limited mostly in calcareous and high pH soils and causes interveinal chlorosis in younger leaves of cereal crops directly damaging their food factories and thus lowering the overall yield (Barker and Pilbeam, 2015). However, the iron deficiency symptoms in crop plants can be tackled by using iron fertilizers both in soil application and foliar forms to cope with the iron deficiency in these plants. The micronutrient fertilizers not only improve plant health but also been reported to increase micronutrient levels in edible parts of crop plants (De Valença *et al.*, 2017). The combined used of nitrogen and foliar micronutrients is reported to improve nutritional composition of edible parts and increase overall yield of crop (Gomaa *et al.*, 2015; Melash and Mengistu, 2020; Kiran *et al.*, 2021). Combined application of iron and nitrogen has already proven its worth by boosting wheat crop yield and nutritional quality (Wojtkowiak *et al.*, 2017). The iron bioavailability, however, still remains neglected during the course of these modern studies. To check the effect of combined fertilizers effects on grain iron bioavailability, two iron sulphate doses were combined with three nitrogen doses in all possible combinations on forty-four wheat accessions.

MATERIALS AND METHODS

Growing conditions

The experiment was carried out at the field area of the Department of Plant Breeding and Genetics, University of Agriculture Faisalabad, Pakistan. The area is situated at 31° - 44' N, 73° - 06' E and 184.4 m above sea level. The soil type is silt loam with 7.8 pH and 2.04 dSm-1 EC. The area is in semiarid region with organic matter 3.7 mg g-1, nitrogen 0.18 mg g-1, phosphorus is 12.5 mg g-1and potassium 0.09 mg g-1 of dry soil. The metrological statistics during crop season were recorded (Figure 01).

Experimental materials

A total of forty-four wheat accessions including 12 parents and 32 advance lines were used in experiment as listed in Table 1. Out of 12, eight parents were high iron contents and four were higher yielders. The parent genotypes were collected from wheat research institute, Faisalabad and Department of Plant Breeding and Genetics of University of Agriculture Faisalabad. Quality parameters of each accession were measured including grain iron, phytate and bioavailable iron of the grains.

Experimental design

The experiment was laid out in randomized complete block design with three factor factors with three replications. The trial started on November 20th, 2016. Each genotype was sown in one-meter-long row with 0.3 m distance between rows and 0.15 m plant to plant distance. Dibbler was used to plant seeds with two seed in one hole. All the standard agronomic practices were ensured afterwards till the crop harvest.

Experimental treatments

Three doses of nitrogen, namely 75, 100 and 150 kg ha-1 were applied. The nitrogen application was in two equal splits i.e. one at sowing time and other with fifteen days interval in form of urea. While iron sulfate ($FeSO_4.7H_2O$ >99%

Table 1. List of genotypes used in the study.

Sr.no	Codes	Genotypes	Sr.no	Codes	Genotypes
1	L1	Sarsabz	23	C11	Iqbal-2000 / PB-2011
2	L2	9884	24	C12	Iqbal-2000 / FSD-2008
3	L3	Iqbal-2000	25	C13	Mexipak-65 / Zincol-16
4	L4	Mexipak-65	26	C14	Mexipak-65 / AAS-11
5	L5	9886	27	C15	Mexipak-65 / PB-2011
6	L6	NARC-2009	28	C16	Mexipak-65 / FSD-2008
7	L7	SKD-1	29	C17	9886 / Zincol-16
8	L8	Anmol-91	30	C18	9886 / AAS-11
9	T1	Zincol-16	31	C19	9886 / PB-2011
10	T2	AAS-11	32	C20	9886 / FSD-2008
11	T3	PB-2011	33	C21	NARC-2009 / Zincol-16
12	T4	FSD-2008	34	C22	NARC-2009 / AAS-11
13	C1	Sarsabz / Zincol-16	35	C23	NARC-2009 / PB-2011
14	C2	Sarsabz / AAS-11	36	C24	NARC-2009 / FSD-2008
15	C3	Sarsabz / PB-2011	37	C25	SKD-1 / Zincol-16
16	C4	Sarsabz / FSD-2008	38	C26	SKD-1 / AAS-11
17	C5	9884 / Zincol-16	39	C27	SKD-1 / PB-2011
18	C6	9884 / AAS-11	40	C28	SKD-1 / FSD-2008
19	C7	9884 / PB-2011	41	C29	Anmol-91 / Zincol-16
20	C8	9884 / FSD-2008	42	C30	Anmol-91 / AAS-11
21	C9	Iqbal-2000 / Zincol-16	43	C31	Anmol-91 / PB-2011
22	C10	Iqbal-2000 / AAS-11	44	C32	Anmol-91 / FSD-2008

pure) was used as iron source. The recommended iron fertilizer of 12kg ha-1 was prepared in 2% and 4% concentration. It was applied at milking stage of crop growth (Hafeez *et al.*, 2021).

Grain yield

The crop duration was 148 days, and it was harvested manually. Three guarded plants from each accession were used for data collection of morphological traits. The spike was also thrashed by hand in cereal laboratory and weight of grains was measured by using electronic weighing balance to estimate grain yield plant^{-1}.

Grain iron contents

Micronutrient analysis were carried out in Allelopathy and Hitech Lab of University of Agriculture Faisalabad. Grain samples were wet digested to recover total iron contents. For wet digestion, 0.2g dried flour samples were taken in conical flask and added with 5ml digestion mixture (HNO_3: $HClO_4$ in 2:1 ratio). After giving a rest of 30 minutes, this mixture was heated on hot plate (250 °C) till the final digestion of samples and solution becomes clear. This clear solution was then diluted to 50ml with deionized water and filtered with Whatsman filter paper No. 42, to store in plastic bottles. Jones and Case method was used to estimate the absorption percentage using Atomic absorption spectrophotometer (Jones Jr and Case, 1990). The international standard of AOAC-1990 was followed to get accurate results (OAC, 1990). The analysis was repeated thrice to get average value. The iron contents were estimated by using standard curve of known absorption intensity. Standard solutions were prepared by commercially available stock solution "AppliChem" in 1000 ppm. The working solutions were prepared by adding deionized water to the stock solution in different concentrations. Glass wear was disinfected by soaking them overnight in 8N HNO3. They were washed afterwords with no reactive deionized water. The absorption readings of standard samples were plotted in excel to get a standard curve. The sample values were extracted using this standard curve and regression equation. The final values were further multiplied with dilution factor to estimate actual iron contents of each sample.

Grain phytate (PA)

Haug and Lantzsch method was used to determine grain phytate contents (PA) in mg g-1 (Haug and Lantzsch, 1983). A fine powder of grain flour weighing 0.02 g was taken in a test tube and added with 10 ml of 0.2N HCl solution and shook for half an hour. Then 0.5 ml of extract was pipetted into in screwed cap test tube and added with one ml ferrous solution. Ferrous solution was prepared by adding 0.2 grams of $Fe(NH_4)_2(SO_4)_2.6H_2O$ with 100 ml 2N HCl and made up to 1000 ml. This solution was heated in water bath for 30 minutes and cooled in ice water for 15 minutes and let it cool to room temperature. Then add 2 ml of 2,2-bipyridine solution was added and mixed. The bipyridine solution was made by adding 10 g of 2,2-Bipyridine with10 ml of thioglycolic acid and added with deionized water to make a final volume of 1000 ml. The absorbance was measured within 30-60 seconds of adding bipyridine solution in the mix and reading of spectrophotometer was noted at 519 nm counter to deionized water. The hue of the mix fades with time due to reaction of bipyridine with iron phytate of the sample. The reference solution was also prepared accordingly. The standard solution's reading on spectrophotometer was used to plot a standard curve and regression equation. This regression equation was then used to determine the phytate of each sample. Three values were recorded and average was used for statistical measurement.

Bioavailable grain iron

To determine the bioavailable grain iron contents of any sample, molar ratio of phytate to iron was calculated. PA/Fe molar ratio was inversely related to bioavailability of iron. Thus, ratio ≤1 was desirable (Hurrell and Egli, 2010). Higher the amount of iron (Fe) lower will be the ratio and more will be the availability of iron. Similarly, a higher ratio represents more phytate in sample, which act as antinutrient and results in reduced bioavailability of grain iron.

Statistical analysis

All the statistical analysis was performed using software. Analysis of variance (RGD, 1997) was done using IBM SPSS 23 and mean determination. The effect of fertilizers were dissected using LSD via Statistix 8.1. Then means were plotted by Microsoft excel 2013. At the end all the genotypes were plotted for all the traits in best fertilizer combination to identify best genotypes performing better in that combination using Graphpad prism 6.

RESULTS

Genetic variability

Significant genetic variability was observed in analysis of variance for all the traits except grain phytate contents (Table 02). For grain phytate contents only genotypes differed considerably and some interactions indicating that genotypes differ for this trait based on their genetic makeup. There is no fertilizer influence for final expression of this trait demonstrating its expression being rigidly under genetic control. While grain yield and other traits are highly influenced by fertilizer application. Grain iron contents showed more effect of iron fertilizer, indicating that grain iron contents can directly be improved by iron fertilizer application.

Recommended fertilizer

Least Significant differences (LSD) were checked for interaction effects of all the factors (Table 03). Highest nitrogen (150 kg ha^{-1}) along with lower iron dose (2%) proved best for lowering grain phytate contents and making more bioavailable iron in grains. However, for grain yield, moderate nitrogen dose (100 kg ha^{-1}) combined best with lower iron dose (2%) to provide favorable results.

The optimal fertilizer combination was identified by plotting mean performances of each trait against all fertilizer combinations (Figure 2). Third fertilizer combination (150 kg ha^{-1} nitrogen and 2% foliar iron) stands as the most suited to improve grain iron contents and their bioavailability without compromising grain yield as described in Figure 2. Highest iron (89.1 μg g^{-1}), phytate (1.9 mg g^{-1}) and phytate to iron molar ratio (3.6) of grains was observed in combination of highest doses of both the fertilizers.

Maximum grain yield (19.5 g) was observed in lowest dose of nitrogen (75 kg ha^{-1}) and highest dose of iron (4%) followed by 19 g in highest nitrogen (150 kg ha^{-1}) and lowest iron (2%) combination. This may reveal an antagonistic relation of both fertilizers in determining grain yield. The lowest phytate (0.9 mg g^{-1}) and molar ratio (1.2) of phytate to iron was observed in third (nitrogen =150 kg ha^{-1} and foliar iron = 2%) fertilizer combination indication best availability of iron among all the experimental units. This combination not only shows highest availability and considerable grain yield but also came up with second highest grain iron contents (61.5 μg g^{-1}). Thus, this combination can be recommended for agronomic biofortification of iron in wheat.

Better performing genotypes

Genotypes performed differently at each fertilizer combination. After identifying the best fertilizer doses, genotypes were plotted by their means for each trait (Figure 03). Some genotypes including C2, C6, C10 and C20 out stand all others in bioavailability of iron due to lower phytate contents. Elevated grain iron concentrations were detected in several genotypes (notably L3, C14, and C29). Higher grain yield was recorded in genotypes such as C6, C10, and C14. Consequently, genotypes C6 and C14 emerge as strong candidates for selection due to their collective advantages of lower grain phytate content (0.6-1 mg g^{-1}), higher yield (14.9-24 g plant^{-1}) and enhanced iron accumulation (80-85 μg g^{-1}), along with superior iron bioavailability (PA/Fe < 1).

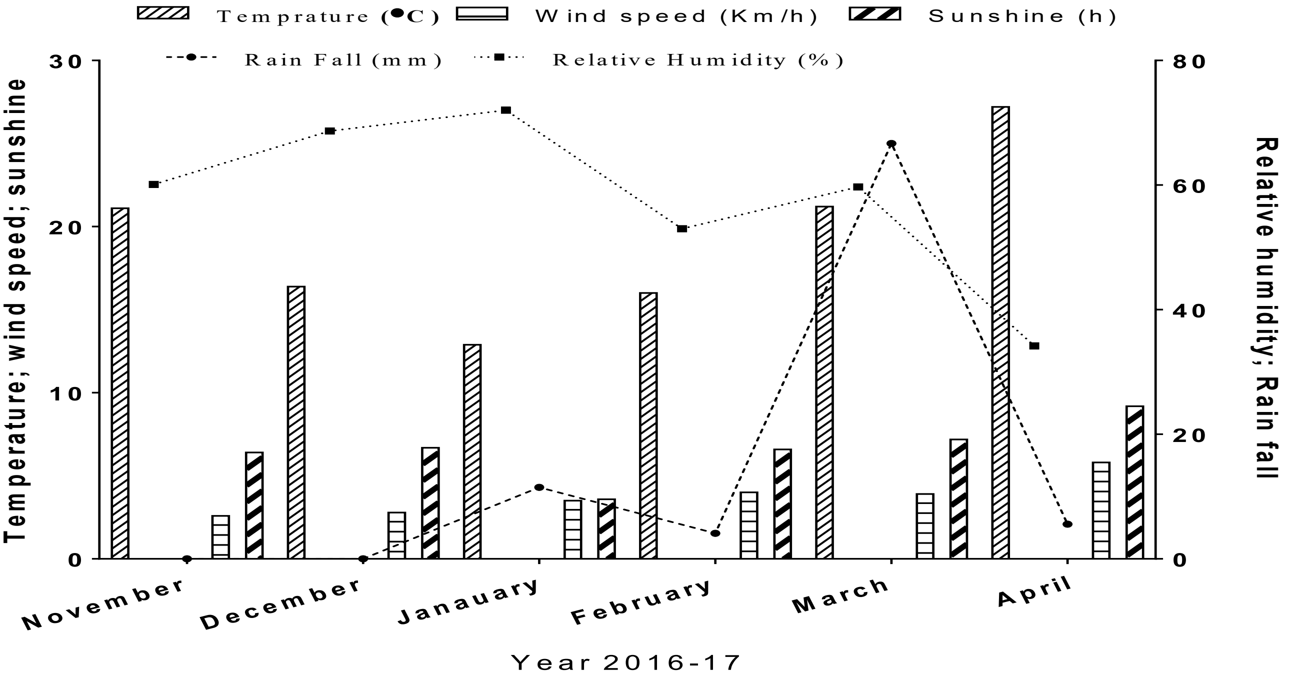

Figure 1. Weather data during wheat growing season (2016-17) at experimental site.

Table 2. Mean squares for the influence of nitrogen and iron on grain yield and grain iron related traits in wheat under influence of nitrogen (N) and iron (Fe) fertilizers.

SOV	Degree of freedom	Grain yield plant^{-1}	Grain phytate (PA)	Grain iron (Fe)	PA/Fe
Replication	2	120.5	0.412	1540.2	6.8
Error	174	45.3	0.213	210.6	1.12
Nitrogen (N)	2	542.4**	1.499	12839.8**	40.3**
Iron (Fe)	1	731.2**	0.298	24404.5**	32.2**
Genotypes (G)	43	110.9**	1.374**	621.1**	5.5**
Fe × N	2	764.7**	38.403**	6848.7**	217.7**
G × N	86	89.4**	0.686	504.5**	2.3**
G × Fe	43	115.9**	0.292	600.3**	1.4*
Fe × G × N	86	71.4**	1.134**	548.3**	4.7**

** = highly significant ∞ P = 0.01%; * = significant ∞ P = 0.05%

Table 3. Influence of nitrogen and iron fertilization on grain iron contents, grain phytate contents, bioavailable grain iron (PA/Fe) and grain yield of bread wheat genotypes.

Nitrogen application (kg ha^{-1})	Grain phytate (mg g^{-1})		Bioavailable grain iron (PA/Fe)		Grain yield (g)	
	2% $FeSO_4$	4% $FeSO_4$	2% $FeSO_4$	4% $FeSO_4$	2% $FeSO_4$	4% $FeSO_4$
5	1.6a	0.8c	4.3a	1.5c	19.2b	20.6b
100	1.6a	1.3b	2.3b	2.1b	26.9a	15.9b
150	0.9c	1.9a	1.2c	2.6b	18.8b	18.2b
LSD values (p 0.05) (N × Fe)	0.27		0.49		4.82	

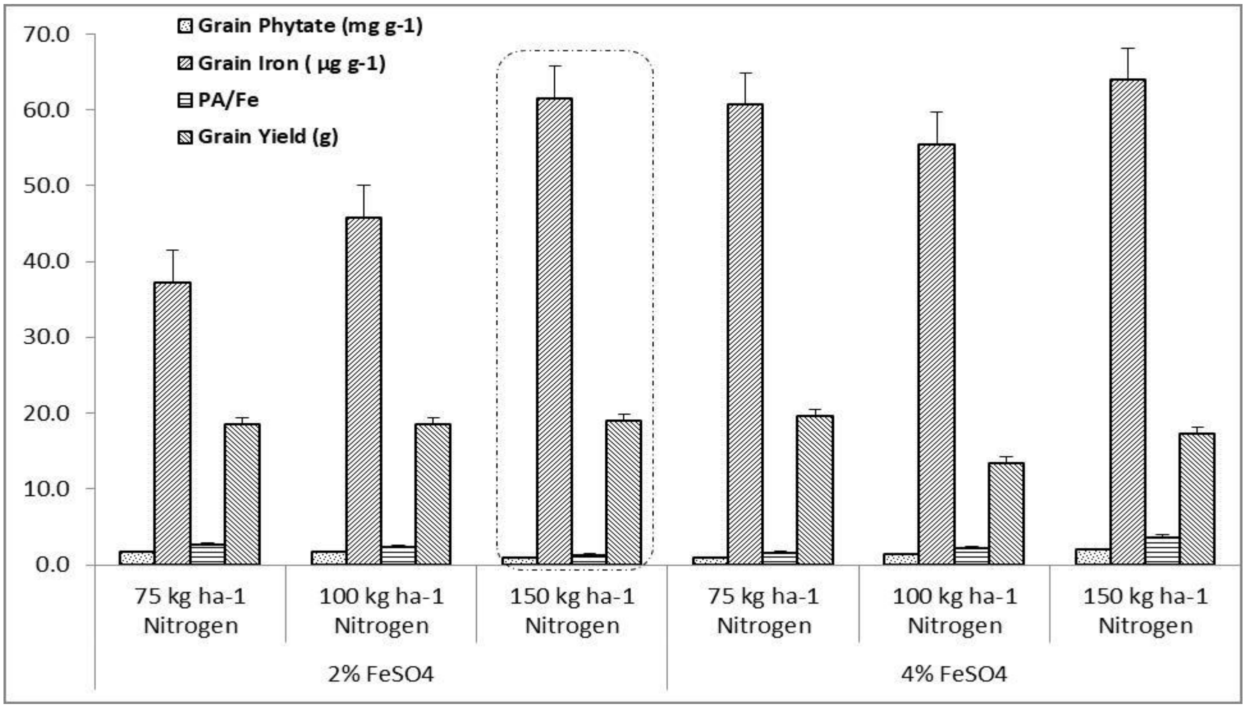

Figure 2. Comparison of fertilizer combination for average values of grain yield, grain phytate contents, grain iron contents and PA/Fe (molar ratio of grain phytate to grain iron).

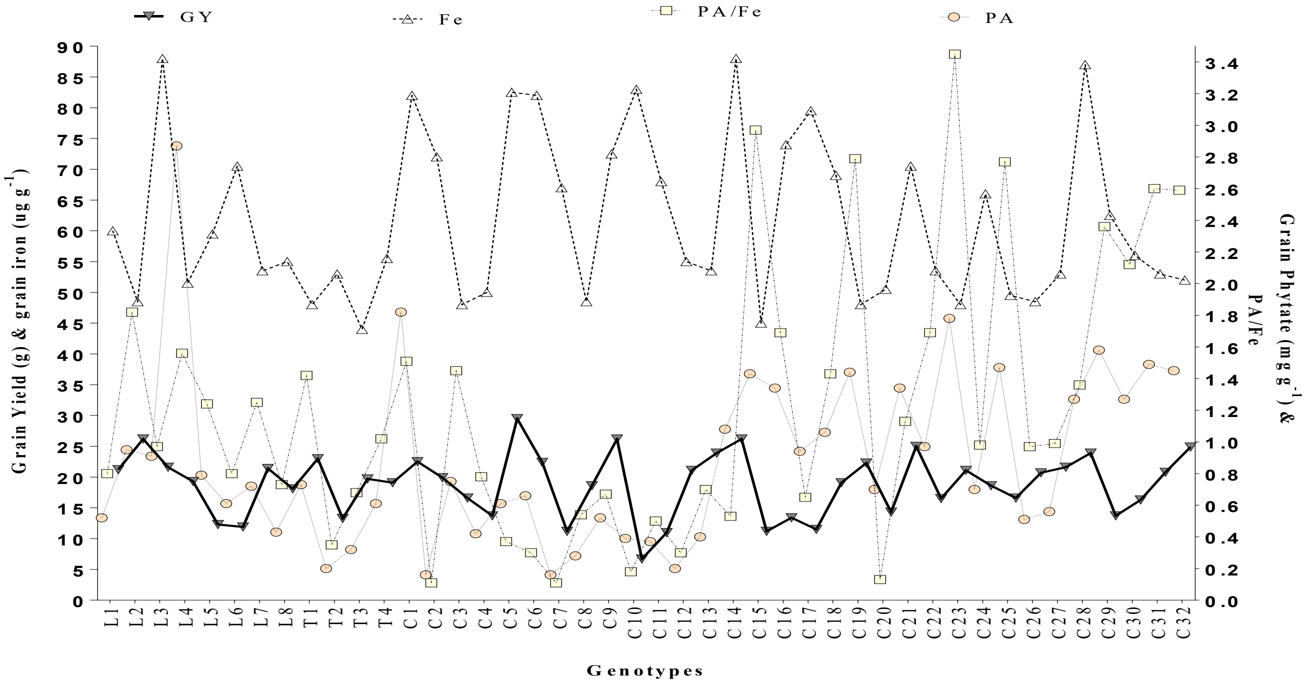

Figure 3. Mean comparison of genotypes in 150 kg ha^{-1} nitrogen and 2% FeSO$_4$ for grain iron (µg g^{-1}), grain yield (g), grain phytate (mg g^{-1}) and PA/Fe (molar ratio of grain phytate to grain iron).

DISCUSSION

Nitrogen fertilizer is mostly used in almost all field crops to increase their growth and production. Likewise, its use in wheat has increased due to development of wheat cultivars that are more responsive to nitrogen fertilizers. Nitrogen makes structural and functional contributions to the crop plants by its being part of chlorophyll, amino acids, ATP and other structural and functional proteins. While iron, being micronutrient, actively participates in plants enzymatic activities due to its catalytic ability and results in optimal plant growth and production. In this study, the individual and combined effects of iron and nitrogen were evaluated to enhance grain yield and quality. This study unfolds the worth of combined application of macro and micro element specifically nitrogen and iron. The fertilizer works in complementation to not only improve grain yield but also improve grain iron. The results indicated that agronomic biofortification using fertilizer combinations is possible by identifying right doses combination and identifying genotypes that respond more to these fertilizers. This will not only improve value of wheat products but will ultimately elevate the iron levels in human population.

Grain yield plant^{-1} was increase more in moderate nitrogen dose and decreases by further increasing nitrogen dose. This may be due to nitrogen losses due to ammonia volatilization, denitrification, surface runoff and leaching into the soil. Despite all the nitrogen losses, the crop plants were actively growing and their vegetative growth was much pronounced. It appears that excessive nitrogen availability at higher application rates disrupted the phenological cycle, causing the plants to remain in a prolonged vegetative state and hindering proper and timely completion of the reproductive phase. Higher iron doses also decreased the grain iron contents compared to lower iron doses. Intensification of chlorophyll contents and photosynthetic process by integrating iron in iron-Sulphur protein of thylakoid membrane in leaves due to application of foliar iron have resulted in increasing grain yield but the excessive iron at higher does may have been stored in vacuoles and not properly utilized in grains of plant (Sultana *et al.*, 2018).

Most of the phytate contents are stored in bran layer of seeds and a minute quantity is found in endosperm. Most of the iron is also found in bound form with phytic acid in protein storage vacuoles that are mostly located in aleurone layer of grain and embryo. Higher nitrogen dose has resulted in more grain iron and phytate quantity in grains may be due to a greater number or bigger size of the grains. All the structural and functional proteins are made up of nitrogen, even phytosiderophores are also nitrogenous compounds and cereal plant roots release them to capture iron from soil (Denton-Thompson and Sayer, 2022). Higher nitrogen doses may have enhanced the quantity of these phytosiderophore compounds to increase iron absorption by roots. The phytase, another enzyme that is responsible for making iron bioavailable, may have improved in higher nitrogen doses to improve iron bioavailability.

More grain iron contents in higher doses of both the fertilizers may be the result of vigorous vegetative growth of plants and more leaves that absorbed more iron to utilize it more efficiently in grains. However, vigorous plants in this fertilizer mix also contained more amount of phytate contents that made grain iron less available. The lower phytate contents in higher nitrogen and low iron dose may be due to soil acidity. Sometimes higher nitrogen application makes soil acidic and bound free phosphorus in soil resulting in less phosphorus absorption by roots. These phenomena may have lowered the phytate and ultimately phytate to iron molar ratio to improve iron bioavailability for human digestion. However, to confirm this hypothesis soil studies are recommended before fertilizer application. Some studies have confirmed higher nitrogen doses to improve bioavailability of grain iron contents (Gomaa *et al.*, 2015).

Each genotype responded differently for each trait under consideration. To understand these differences molecular studies are to be carried out to identify the gene expression and its changes under different fertilizer regimes. The genotype and environmental interactions may be the actual culprit behind these differential behavior genotypes for fertilizer combinations. In selected fertilizer doses, some genotypes performed better for iron contents of grains, bioavailability of iron and phytate in grains all at once, indicating some shared genetic material and regulatory mechanisms of all these characters. A few genomic materials are recognized in previous studies for controlling grain iron profiles in wheat. These genomic areas are situated on 5A, 5B and 7B chromosomes. While 5B is also known to control phosphorus quantity in plants ultimately determining the phytate contents of grains (Cu *et al.*, 2020). Fertilizers are also known to alter gene expressions. Some genes are upregulated by fertilizer application and others are down regulated. Foliar iron salt sprays also have proven their part solely and in conjugation with other fertilizers by improving grain iron contents (Ramzani *et al.*, 2016; Melash and Mengistu, 2020; Kiran *et al.*, 2021). Therefore, genotypes like C6 and C14 can be used to develop cultivars and can also be recommended for cultivation under specified fertilizer management to improve iron profiles with increased yield. Ultimately solving iron deficiency problem in human population. This would specifically benefit the low-income groups of population who cannot afford iron supplements in surviving iron deficiency.

CONCLUSION

Agronomic iron biofortification of wheat is possible by using a combination of fertilizers. The combined application of 150 kg ha^{-1} nitrogen and a 2% foliar spray of iron sulphate at the milking stage was found to be highly effective. At this fertilizer combination, grain iron and its bioavailability improves by decreasing the phytate contents without effecting grain yield. Although most genotypes responded positively to this fertilizer combination, notable variations in performance were still observed, likely attributable to underlying genetic differences. Genetic study for improving iron and its bioavailability can reveal the differential expression of these genotypes in response to different fertilizer regimes.

AUTHOR CONTRIBUTIONS

Abia Younas: Conducting experiment and writing original draft. Muhammad Farooq and Muhammad Kashif: Experiment concept and layout. Sundas Waqar and Asma Parveen: Writing, editing, and statistical analysis. Iqra Ghafoor and Mehwish Makhdoom: Statistical analysis and reviewing. Muhammad Uzair: Proof reading and reviewing.

CONFLICT OF INTEREST

There is no potential conflict of interest in the research and manuscript.

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