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Nitrogen nutrition of crops: A critical determinant of plant iron and zinc uptake and their biofortification in grains of wheat

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Abstract
Declining soil fertility, imbalanced and non-judicious application of major fertilizers, decreasing crop productivity and produce quality and malnutrition among burgeoning human population are some of the major challenges of the present time. An experiment was conducted to assess role of N nutrition of wheat crop on efficiency for plant micronutrient uptake and their subsequent accumulation in the grain. The study involved measuring the effect of varying level fertilizer N application (N0, N120, N150 and N200 kg N ha^{-1}) on root uptake, shoot partitioning and grain accumulation of iron (Fe) and zinc (Zn) in two bread wheat cultivars HD 2967 and DPW 621-50. N fertilizer application, in general, bettered plant vigor as evident from high root and shoot mass, root surface area and flag leaf area of N fertilized plants than those maintained without N. Fertilizer N application improved plant N status and shoot and grain concentration of Fe and Zn, more so in a dose dependant manner. Root to shoot and shoot to grain translocation efficiency for Fe and Zn increased with increasing availability of soil N. Higher biomass production with N fertilization led to a dilution effect on Fe and Zn concentration and caused a reduction in nutrient use efficiency for Fe, Zn and N with N than without N treatment. The concentration of these microelements in wheat shoot and grain increased with increasing N fertilizer application, reaching the highest at N150. Further, increase in N availability did not significantly improve the grain micronutrients. These results indicate that fertilizer N management of crops is critical determinant of Fe and Zn accumulation in the grain. Improved sink demand and efficient retranslocation of micronutrients at high N availability could be the underlying operative mechanisms responsible for agronomic biofortification.

Keywords: Soil nitrogen, grain iron and zinc, nutrient use efficiency, translocation index, wheat

Introduction
More than two billion people, mostly the children below the age of five and pregnant women, worldwide are affected by dietary deficiency of essential micronutrients such as iron (Fe) and zinc (Zn) affects (White and Broadley, 2009; WHO, 2012) [38, 39]. In many parts of the world, micronutrient deficiency is a more widespread problem than low energy intake and poor dietary quality (Stewart *et al*., 2010) [33], and about 20% of deaths in children under five can be attributed to vitamin A, Zn, Fe, and/or I deficiency (Prentice *et al*., 2008) [29]. In countries with a high incidence of micronutrient deficiencies, cereal-based foods represent the largest proportion of the daily diet (Bouis *et al*., 2011; Cakmak *et al*., 2010) [5, 9]. The Harvest Plus initiative taken up by the CGIAR consortium is working with national and international partners to alleviate mineral nutrient deficiencies by bio-fortifying staple food crops with essential minerals and vitamins; an approach which is considered to be the most economical solution to micronutrient deficiency (Welch and Graham, 2004; Bouis, 2009; Peleg *et al*., 2009) [36, 5, 27].

Cereals such as wheat and rice are the most important sources of calories in many countries and increasing the Zn and Fe concentrations of cereal grains, thus, is currently a high-priority research area (Bouis, 2003; Cakmak, 2008; White & Broadley, 2009) [38, 6, 8]. However, their uptake and accumulation in grains is governed by several physiological, agronomic and molecular attributes. Also widespread deficiency of essential mineral nutrients and their imbalance in the soil and interactions therein further inhibit the availability of macro and micro nutrients for plant uptake and subsequent translocation to the shoot and to the grain. Plants exhibit several adaptive strategies to improve the amount of available mineral nutrients in their rhizosphere (Cheng *et al*., 1995) [10]. Further, recent literature indicates that the combination of physiological and agronomic biofortification with breeding is a practical and sustainable approach to the Zn-deficiency problem in humans (Pfeiffer and McClafferty, 2007; Cakmak, 2008) [28, 8].

Micronutrient analysis of more than 3000 germplasm accessions, including hexaploid, tetraploid, and diploid sources from the
International Maize and Wheat Improvement Center (CIMMYT) gene bank reveals huge genetic variability for Zn and Fe content in wheat (Monasterio and Graham, 2000) [25]. Research also suggests that an optimum management of crop irrigation and fertilizer application schedule may help to improve plant micronutrients (Graham *et al*., 1999) [16] for eg. Suitable nitrogen application promoted Fe, Mn, Cu and Zn accumulation in grains of rice/wheat (Cheng *et al*., 1995, Yuan *et al*., 2005) [13]. However, the effect of soil N availability on distribution of Fe, and Zn in whole shoot and grain of wheat is not clear. It is assumed that manipulation of N nutrition can have a significant effect on retranslocation of Fe and Zn in cereals and a positive effect is postulated because N is required for the biosynthesis of nicotianamine (NA), a major chelator for Fe during retranslocation via phloem (Von Wirén *et al*., 1999) [35] and the iron transport peptide (Kruger *et al*., 2002, Shi *et al*., 2012). Additional supply of N is suggested to enhance Fe and Zn accumulation in wheat grains (Erenoglu *et al*., 2011, Shi *et al*., 2012) [14]. We propose a hypothesis that nitrogen management of crops is important for improving plant and grain micronutrients. In the present study, two high yielding bread wheat cultivars were evaluated for fertilizer N response under field condition to ascertain the relationship between N application, total plant micronutrient (Fe and Zn) uptake and grain accumulation of Fe and Zn to confirm the above hypothesis.

Materials and methods

Planting material and experimental setup

Grains of two high yielding bread wheat cultivars, HD 2967 and DPW 621-50 were obtained from Division of Genetics and Plant Breeding, IARI, New Delhi and raised in field soil in pots at different levels of applied N to assess latter's role in iron and zinc uptake and mobilization to the shoot and the grain. Physicochemical properties of the experimental soil were as follows: pH 8.25; electrical conductivity 0.32 dSm^{-1}; organic carbon 0.45%; available N 165.3 kg ha^{-1}, available P 12.2 kg ha^{-1}, available K 239.5 7 kg ha^{-1}, available Zn 0.72 mg kg^{-1}, available Fe 3.80 mg kg^{-1}. Plants were raised in circular plastic pots of 18" dia in open air net house with 30 kg soil per pot at 4 levels of soil N i.e., 0 (Control), 120, 150 and 200 kg N/ha supplied as urea in two splits (70 and 30 per cent as basal and at 1 month stage). Three replicates per treatment were maintained in a completely randomized block design following crop specific irrigation, fertilizer and agronomic schedule. Recommended dose of P @ 60 kg/ha and K of 40 kg/ha were supplied to all the pots irrespective of the N treatments. Observations on different growth and physiological attributes were recorded at 30, 60, 90, 145 (harvest) days after sowing (DAS) if not mentioned otherwise.

Plant biomass, root and leaf area and grain yield characteristics

Plant samples were collected in triplicate at 30, 60, 90 DAS and at harvest for the two experimental wheat cultivars raised under four different nitrogen levels. For this, the potted plants were placed under continuously flowing water for the soil to loosen and to facilitate the removal of plant with root and to minimize damage to the root system. The roots were thoroughly washed and plant was separated into shoot and root tissues and kept for drying in a hot air oven at 65 °C till constant tissue weights were obtained. After complete dryness, shoot and root biomass were recorded. Flag leaf area as affected by soil N status was measured at 80 DAS, using LICOR LI-3100 Leaf Scanner and expressed as cm^{-2}. Root surface area was determined following Ansari *et al*., (1995) by carefully removing the plant roots. Roots were thoroughly washed to remove soil particle, drained of excess water by gently tapping them between the folds of filter paper and then immersed completely upto root/shoot junction in a known volume of 0.05M of $NaNO_2$ solution for 10 seconds. Roots were dried in air to drain excess of $NaNO_2$ solution and transferred to a beaker containing a known amount of distilled water for 15 min under regular stirring. Plants were removed and nitrite level of the distilled water which essentially comes from the root surface was measured by pippetting a suitable volume of aliquot in to the test tube and developing the color by adding 1 ml 1% sulphanilamide and 1 ml 0.01N NEDD. Color was allowed to develop for 20min at RT and the total volume was made up to 4 ml with dH_2O. The nitrite content was determined by measuring the absorbance at 540 nm and the absorbance value was used to calculate root surface area using the following two factor combination i.e., 1OD=127µmol NO_2 and 100µmol NO_2 =33 cm^2 root surface area.
Further, grain yield attributes viz., total grain yield per plant and seed test weight were measured at the harvest stage.

Gas exchange attributes

Effect of N application on gas exchange characteristics i.e., photosynthetic rate (A), transpiration rate (Tr) and stomatal conductance (Gs) were measured in wheat using portable Infrared Gas Analyzer (IRGA), LI-6400-F Model (Li-COR Ltd., Lincoln, Nebraska, USA) at 30, 60, 90 DAS between 10 to 11.30 am in the fully open leaf of plants. The leaf to be analyzed was placed in the leaf chamber and differential for CO_2 and moisture concentration in the chamber was taken as the measure of CO_2 assimilation and transpiration rate respectively. Photosynthetic rate (A), stomatal conductance (g_s) and transpiration rate (T_r) were expressed as µmol (CO_2) $m^{-2}s^1$, mol CO_2 $m^{-2}s^{-1}$ and mmol H_2O $m^{-2}s^{-1}$ respectively.

Activity of Key Enzymes of N metabolism

In vivo activity of nitrate reductase (NR) and Glutamine synthetase (GS) were measured in fully opened leaf at 30, 60 and 90 DAS as follows. NR (EC 1·6·6·1) activity was estimated using the method of Klepper *et al*., (1971) as modified by Nair and Abrol (1973). The nitrite produced in the reaction was estimated by the method of Evans and Nason (1953). Absorbance was measured using a UV-Vis spectrophotometer (UV57045S) at 540 nm. The calibration curve was prepared using standard sodium nitrite solution. The enzyme activity was expressed as µmol nitrite formed g^{-1} fresh mass h^{-1}. *In vitro* GS activity (EC 6.3.1.2) was assayed following the method of Mohanty and Fletcher (1980). The GS activity was calculated from the standard curve of γ-glutamyl hydroxymate as the amount of ferric γ-glutamyl hydroxymate formed and expressed as µmol γ- glutamyl hydroxymate formed g^{-1} fresh mass h^{-1}.

Mineral nutrient analysis

Tissue mineral nutrients chiefly, N, Fe and Zn were measured in shoot, root and grains at assorted stages of growth from 30 DAS to harvest. Respective plant tissues were oven dried at 65 ^{0}C until complete dryness and were acid digested as stated below to determine their N, Fe and Zn level. For Fe and Zn, a

known mass (100-200 mg) of the dried root, shoot and grain samples were digested in the di-acid mixture (nitric acid: perchloric acid 9:4) and subsequently analyzed for Fe and Zn in Atomic Absorption Spectrophotometer (AAS, ECIL, India). Tissue nitrogen content was estimated by Kjeldahl Method using the Kjeltec System 1002. Grain protein concentration was determined by multiplying the respective grain N (%) value with a factor of 5.81.

Translocation index (TI) for Fe and Zn

Root to shoot translocation of Fe and Zn, as affected by N availability, in wheat was calculated using the following formula.

TI(Fe) = (Shoot Fe/Plant Fe)

TI(Zn) = (Shoot Zn/Plant Zn)

Nutrient use efficiency (NUE)

Physiological plant nutrient (N, Fe and Zn) use efficiency was measured in terms of capacity for shoot biomass production per unit of shoot nutrient uptake; summarized as follows:

NUE^{Fe} = Shoot biomass / **S**hoot Fe

NUE^{Zn} = Shoot biomass / **S**hoot Zn

NUE^{N} = Shoot biomass / **S**hoot N

Statistical analysis

The significance of the effect of the treatments and their interactions on the reported traits was evaluated by Two-Way Analysis of Variance (ANOVA) using SPSS 16.0. The critical difference (CD) at 0.05 probability ($P<0.05$) was worked out for each parameter.

Results and discussion

Effect of fertilizer N application on plant growth and yield attributes

Shoot mass, across the experimental wheat cultivars, increased with crop age and N application. Shoot biomass increased significantly at N120 up to N150 when compared with N0 (control). Although shoot accumulated a higher dry mass at N200 than N0, it was insignificantly greater than that measured at N150. Root mass showed a similar pattern of variation with respect to N treatment, however, the magnitude of variation between N treatments was lesser than that observed for the shoot mass (Table 1). Root mass irrespective of the stage of growth, increased with an increase in N application until N150 followed by an insignificant decline for both the experimental cultivars. Root: shoot ratio decreased with increasing N levels in both the cultivars (Table 1). Further, an incremental increase in leaf area in the flag leaves of both the cultivars was observed with increasing levels of N (Figure 1). Highest flag leaf area of 36.9 cm^2 and 35.0 cm^2 in DPW 621-50 and HD 2967 respectively was observed at N200. Increase in crop growth characteristics with N fertilization is widely reported (Liu *et al*., 2014).

Root surface area, measured at 30, 60 and 90DAS, increased with crop growth and with increasing N fertilization in both the experimental cultivars (Figure 2) more so in a dose dependant manner. Cultivar differences for root surface area, when averaged over the fertilizer nitrogen treatments, were insignificant. Increase in root characteristics in response to N fertilization may be caused by induction of NRT1.1 which is a transceptor (Sun and Zheng, 2015) [34] with dual role and affinity i.e., as nitrate transporter and as nitrate receptor and as both high and low affinity transporter. NRT1.1. is shown to regulate auxin movement out of the root to trigger growth of lateral roots in the presence of N fertilizer and consequently facilitates N uptake (Krouk *et al*., 2010) [19].

Grain yield, in general and across the two cultivars, increased with an increase in N application when compared with N0. However, this increase in yield was insignificant at fertilizer N dose >N150. N response of the experimental cultivars in terms of grain production on per plant basis was identical. Grain test mass was significantly reduced at N0 than with N application (Table 5). An average increase of 40 per cent in grain mass was measured across the experimental cultivars at N150 when compared with grain mass recorded at N0. Results clearly reveal that plant response to N application measured in terms of grain yield and grain filling i.e., grain mass declines at N supplies beyond N150. The above results are in agreement with other pot and field experiments with wheat (Hossain *et al*., 2008; Weligama *et al*., 2010) [17, 37].

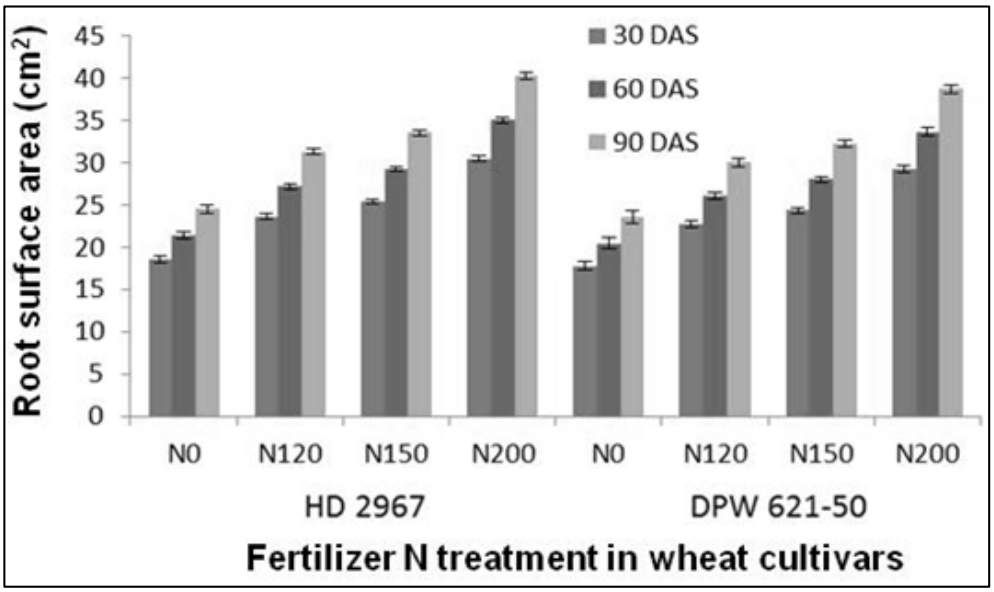

Fig 1: Effect of fertilizer nitrogen application on root surface area of wheat cultivars HD-2967 and DPW-621-50 at different stages of crop growth

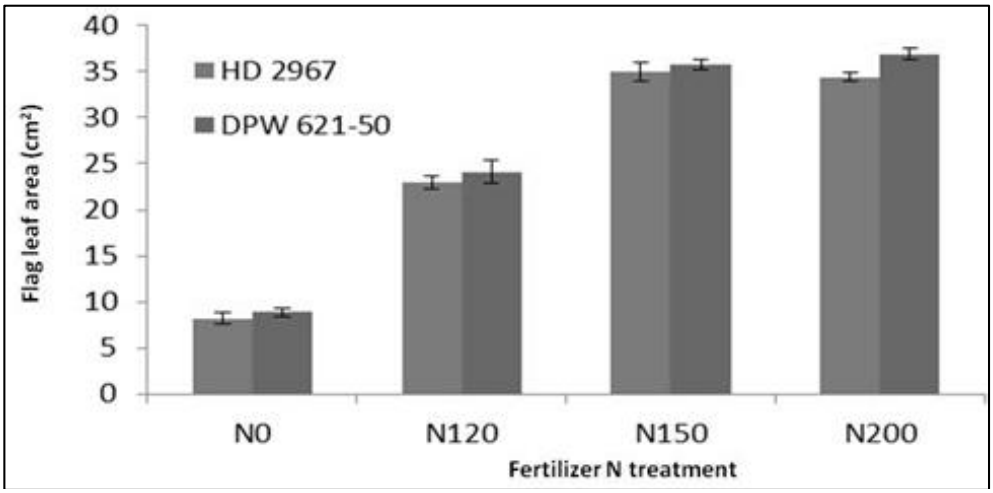

Fig 2: Effect of fertilizer nitrogen application on grain N (%) and grain protein content (%) in wheat

Table 1: Effect of fertilizer nitrogen application on shoot and root biomass and root: shoot ratio of wheat cultivars HD2967 and DPW-621-50 at different stages of crop growth

Wheat Cultivar (C)	Treatment (N)	Days After Sowing (DAS)			
		30	60	90	Harvest
Shoot biomass (g dw)					
HD 2967	N0	0.422	7.36	13.49	13.98
	N120	0.524	9.12	16.06	16.77
	N150	0.639	9.89	18.66	19.58
	N200	0.547	9.35	17.49	18.22
	Mean	0.533	8.93	16.42	17.14
DPW-621-50	N0	0.533	8.01	13.64	13.75
	N120	0.438	8.95	17.68	17.81
	N150	0.490	10.97	19.15	19.40
	N200	0.660	10.25	18.84	18.98
	Mean	0.530	9.54	17.33	17.48
CD @ 5%	C	0.009	0.13	0.11	0.13
	N	0.012	0.18	0.15	0.18
	C X N	0.017	0.25	0.21	0.26
Root biomass (g dw)					
HD 2967	N0	0.215	1.38	2.63	2.46
	N120	0.242	1.55	2.87	2.72
	N150	0.307	1.77	3.31	3.14
	N200	0.220	1.63	3.16	3.05
	Mean	0.246	1.59	2.99	2.84
DPW-621-50	N0	0.258	1.59	2.70	2.92
	N120	0.290	1.76	3.30	3.18
	N150	0.367	2.04	3.81	3.61
	N200	0.264	1.84	3.63	3.49
	Mean	0.295	1.81	3.36	3.30
CD @ 5%	C	0.012	0.08	0.13	0.11
	N	0.017	0.11	0.19	0.16
	C X N	0.024	0.15	0.27	0.22
Root : Shoot					
HD 2967	N0	0.511	0.188	0.195	0.176
	N120	0.461	0.170	0.179	0.162
	N150	0.480	0.179	0.177	0.160
	N200	0.402	0.175	0.181	0.167
	Mean	0.464	0.178	0.183	0.166
DPW-621-50	N0	0.589	0.199	0.198	0.213
	N120	0.593	0.196	0.187	0.179
	N150	0.556	0.186	0.199	0.186
	N200	0.433	0.180	0.193	0.184

	Mean	0.543	0.190	0.194	0.190
CD @ 5%	C	0.021	0.008	0.008	0.007
	N	0.030	0.012	0.011	0.010
	C X N	0.043	0.017	0.016	0.014

Effect of soil N supply on leaf gas exchange characteristics (A, g_s and Tr)

Leaf photosynthetic rate (A), stomatal conductance (g_s) and transpiration rate (Tr) increased with increasing levels of N fertilization (Table 2). This incremental effect in leaf A is mainly attributed to the supply of N for synthesis of chloroplast proteins. Increase in soil N availability and plant N content improves leaf photosynthesis due to N mediated increase in leaf chlorophyll content and Rubisco activity. Stomatal conductance and transpiration rate, irrespective of the wheat cultivar, also increased with crop duration and with N application in a dose dependant manner. Wheat cv. DPW-621-50 showed marginally better gas exchange traits than HD-2967.

Table 2: Effect of fertilizer nitrogen application on Photosynthetic rate (A), stomatal conductance (g_s) and transpiration rate (Tr) at different growth stages (30, 60 & 90 DAS) in wheat

Wheat Cultivar (C)	Treatment (N)	A (µmol (CO_2) $m^{-2}s^{-1}$)			Gs (mol CO_2 $m^{-2}s^{-1}$)			Tr (mmol H_2O $m^{-2}s^{-1}$)		
		Days after sowing (DAS)								
		30	60	90	30	60	90	30	60	90
HD 2967	N0	10.96	14.36	16.21	0.09	0.25	0.26	2.35	2.76	3.10
	N120	11.32	15.95	18.60	0.09	0.27	0.32	2.44	2.84	3.31
	N150	11.62	17.32	22.34	0.09	0.31	0.36	2.44	3.21	4.43
	N200	12.12	18.43	22.68	0.11	0.33	0.36	2.49	3.31	4.52
	Mean	11.51	16.51	19.96	0.09	0.29	0.33	2.43	3.03	3.84
DPW-621-50	N0	11.49	13.35	18.80	0.09	0.22	0.31	2.38	2.62	3.41
	N120	12.33	15.56	20.28	0.11	0.31	0.33	2.46	2.90	3.97
	N150	12.84	17.88	23.67	0.12	0.31	0.38	2.46	3.28	4.96
	N200	12.78	18.87	24.67	0.12	0.32	0.38	2.52	3.41	5.06
	Mean	12.36	16.41	21.85	0.11	0.29	0.35	2.46	3.05	4.35
CD @ 5%	C	0.07	0.10	0.16	0.01	0.01	0.01	0.01	0.02	0.01
	N	0.10	0.14	0.22	0.01	0.01	0.01	0.01	0.03	0.01
	C X N	0.14	0.20	0.31	0.01	0.01	0.01	0.02	0.04	0.02

Effect of soil N supply on enzymes of N metabolism

Response of N availability in the crop rhizosphere on the activity of key enzymes involved in N metabolism i.e., nitrate reductase (NR) and glutamine synthetase (GS) in leaf at 30, 60 and 90DAS (Table 3). Highest NR activity was measured at 60DAS and at N200 for both the wheat cultivars. A N-dose dependant increase in NR and GS was measured. GS activity increased from 30 to DAS for all the N treatments, but declined at 90DAS at N0 and increased with increasing N application at 90DAS for all other N-supplementation treatments. Like NR, GS also plays a major role in N metabolism i.e., in ammonia assimilation to form the amino acid glutamine. Higher N availability leads to a higher Rubisco activity, which in turn results in production of more carbon skeletons and ATP necessary for ammonium assimilation leading to increase in GS activity. An enhanced activity of GS results in greater recycling of N towards grains at maturity, indicating positive role of assimilate supply in mobilization of N towards grains during maturation.

Table 3: Effect of fertilizer nitrogen application on Nitrate reductase (NR) and Glutamine synthetase (GS) activity at different growth stages (30, 60 & 90 DAS) of wheat cultivars

Wheat Cultivar (C)	Treatment (N)	NR activity (µmol NO_2 g^{-1} FW h^{-1})			GS activity (γ-glutamyl hydroxymate g^{-1} FW h^{-1})		
		Days after sowing (DAS)					
		30	60	90	30	60	90
HD 2967	N0	2.04	1.59	1.62	1.46	3.38	2.65
	N120	2.47	3.90	2.60	2.45	7.25	9.06
	N150	3.03	4.30	3.18	3.13	7.81	9.76
	N200	3.27	4.63	3.43	3.29	8.02	10.02
	Mean	2.70	3.60	2.71	2.58	6.61	7.87
DPW-621-50	N0	2.26	1.40	2.37	1.88	3.26	2.33
	N120	2.91	4.37	3.06	3.14	8.29	10.37
	N150	3.33	4.80	3.50	3.80	9.09	11.36
	N200	3.44	4.89	3.61	4.04	9.55	11.93
	Mean	2.99	3.86	3.13	3.22	7.55	9.00
CD @ 5%	C	0.03	0.04	0.03	0.03	0.06	0.08
	N	0.04	0.05	0.04	0.04	0.09	0.11
	C X N	0.06	0.07	0.06	0.05	0.13	0.16

Effect of soil N supply on shoot, root and grain minerals (N, Fe and Zn)

N content of shoot and root, measured at 30, 60, 90 DAS and at harvest, showed incremental increase in tissue N with increasing N fertilization level in both the experimental wheat cultivars (Table 4) up to N150. Further increase in N levels from N150 to N200 didn't result in any significant increase in shoot and root tissue N concentration. Both shoot and root N

concentration declined with crop growth as evident from a significantly lower N accumulation in shoot and root at harvest than those measured at other sampling stages i.e., 30, 60 and 90DAS. Further, there was a significant increase in grain N and protein content upto N150 in both the cultivars (Figure 3). Increasing N beyond N150 to N200 didn't significantly increase the grain N and protein content across the cultivars. This increase in grain N and protein content can be mainly due to N mediated increase in GS activity as shown in table 3. GS also plays an important role in remobilization of N towards grains during maturation. Increasing the level of N fertilization delays the onset of senescence and increases the amount of remobilized N (Barbottin *et al*., 2005) [3].

Shoot Fe concentration was significantly improved by the increasing N from N0 to N150, while a further increase in N availability i.e., at N200, did not increase the shoot Fe concentration significantly when compared with N150. Under low N condition i.e., N0, cv. HD-2967 accumulated a higher concentration of leaf Fe than cv. DPW-621-50. However, in the presence of N, the latter cultivar gathered more shoot and root Fe than when compared with cv HD-2967. Respective increase of 42 and 38% in shoot Fe concentration was measured for cv. DPW-621-50 and HD 2967 at N150 compared to N0. Root Fe concentration was also positively influenced by increasing N levels (Table 6). Both shoot and root Fe increased with crop duration until 90 DAS followed by a decline at harvest. Latter may hint at retranslocation of nutrients from the senescing plants to the developing grains. Grain Fe and Zn increased with increasing N fertilizer application, reaching the highest at 150 kg N /ha (N150) followed by a insignificant decline at N200. Wheat cultivar HD-2967 bettered the cv DPW-621 in terms of grain accumulation of Fe and Zn (Table 5 and 6).

Plant root, in general, possessed a higher Zn concentration than the shoot. N availability does alter the root uptake and root to shoot translocation of Zn as evident from a significantly lower Zn in the shoot/root at N0 than with N application (N120). N dose higher than 120 kg/ha does not cause an commensurate incremental increase in shoot and root Zn level (Table 7). Both shoot and root Zn significantly increased with increasing nitrogen levels from N0 to N150 however, a further increase in N from N150 to N200 didn't show any significant increase in shoot and root Zn concentration in both the cultivars. Bread wheat cv. DPW 621-50 showed better incremental increase in shoot and root Zn concentration than cv. HD 2967 at all the four growth stages. Grain Zn concentration also increased with increasing N fertilizer levels in both the wheat cultivars (Table 5 and 7). Wheat cv. DPW 621-50 and HD-2967 recorded respectively, 20.2 and 26.2 per cent increase in grain Zn at N150 when compared to control. Grain Zn did not change significantly at N200 as compared to N150. The results suggest that increasing N no doubt improves the uptake of Zn, the positive effect of N on Zn plateaus beyond N150. Recommended application of NPK in rice was shown to improve plant uptake of various micronutrients including Fe and Zn (Shengzhe *et al*., 2005) [13]. However, the increase in shoot and grain Fe and Zn concentration as observed in both the experimental cultivars may not only be due to enhanced acquisition and translocation of Fe and Zn to grains but also due to N stimulated Zn and Fe retranslocation out of flag leaves and other plant organs (Shi *et al*., 2010; Kutman *et al*., 2011, 2012; Erenoglu *et al*., 2011) [14, 21]. It is also mooted that since high N supply increases grain protein concentration, the sink strength of the grains for Fe and Zn is enhanced, leading to an increased accumulation of these micronutrients in the grains.

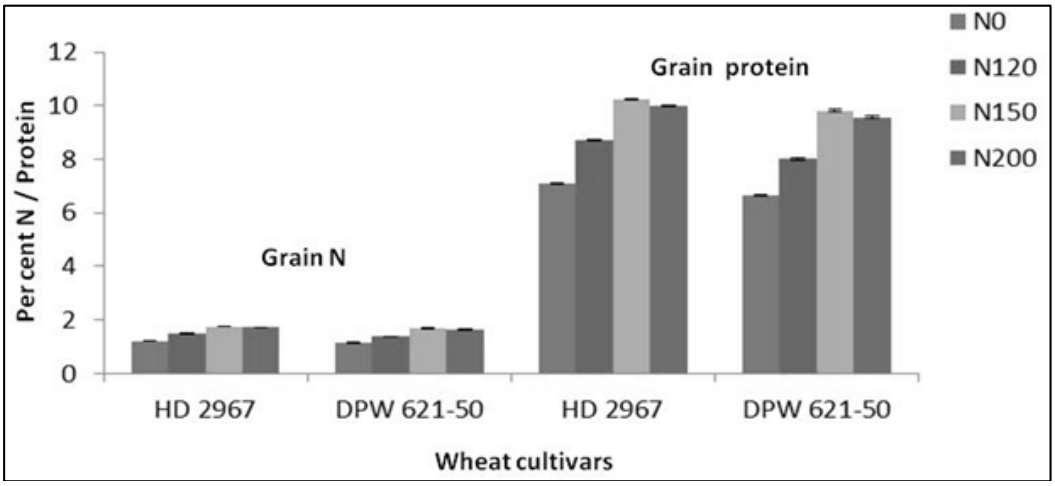

Fig 3: 2 and 3 can also be given as supplementary data

Table 4: Effect of fertilizer nitrogen application on shoot and root nitrogen content at different growth stages in wheat

Wheat Cultivar (C)	Treatment (N)	Days After Sowing (DAS)			
		30	60	90	At Harvest
Shoot nitrogen content (%)					
HD 2967	N0	0.535	0.509	0.410	0.327
	N120	0.798	0.682	0.530	0.402
	N150	0.879	0.761	0.584	0.450
	N200	0.855	0.773	0.607	0.452
	Mean	0.767	0.681	0.533	0.408
DPW-621-50	N0	0.519	0.507	0.423	0.318
	N120	0.752	0.634	0.576	0.406
	N150	0.861	0.792	0.574	0.453
	N200	0.851	0.743	0.609	0.465

	Mean	0.746	0.669	0.546	0.411
CD @ 5%	C	0.007	0.006	0.005	0.004
	N	0.009	0.008	0.007	0.005
	C X N	0.013	0.012	0.009	0.007
Root nitrogen content (%)					
HD 2967	N0	0.418	0.323	0.315	0.314
	N120	0.450	0.358	0.429	0.349
	N150	0.525	0.455	0.498	0.420
	N200	0.565	0.472	0.517	0.428
	Mean	0.489	0.402	0.440	0.378
DPW-621-50	N0	0.418	0.321	0.313	0.309
	N120	0.489	0.384	0.422	0.345
	N150	0.515	0.472	0.505	0.410
	N200	0.535	0.477	0.512	0.417
	Mean	0.490	0.413	0.438	0.370
CD @ 5%	C	0.020	0.004	0.004	0.003
	N	0.028	0.005	0.005	0.005
	C X N	0.040	0.007	0.008	0.006

Table 5: Effect of fertilizer nitrogen application on grain iron, zinc content, test weight and grain yield in wheat

Wheat Cultivar (C)	Treatment (N)	Grain Fe (mg/kg)	Grain Zn (mg/kg)	Test weight (g/1000 seeds)	Grain yield (g/plant)
HD 2967	N0	34.22	30.24	28.69	5.56
	N120	38.36	34.32	36.98	7.17
	N150	44.36	40.97	43.63	8.46
	N200	44.21	40.19	45.67	8.85
	Mean	40.29	36.43	38.74	7.51
DPW-621-50	N0	32.25	29.70	30.21	5.86
	N120	36.59	31.63	37.00	7.17
	N150	42.87	37.25	46.59	9.03
	N200	42.21	37.16	46.28	8.97
	Mean	38.48	33.94	40.02	7.76
CD @ 5%	C	0.10	0.09	0.60	0.12
	N	0.15	0.13	0.85	0.16
	C X N	0.21	0.18	1.20	0.23

Table 6: Effect of fertilizer nitrogen application on shoot and root iron concentration at different growth stages in wheat

Wheat Cultivar (C)	Treatment (N)	Days After Sowing (DAS)			
		30	60	90	At Harvest
Shoot iron (µg g^{-1} DW)					
HD 2967	N0	416.3	489.8	465.3	232.7
	N120	603.9	710.5	675.0	337.5
	N150	674.0	792.9	753.3	452.0
	N200	668.9	787.0	747.6	411.2
	Mean	590.8	695.1	660.3	358.3
DPW-621-50	N0	425.1	500.1	475.1	167.8
	N120	637.2	674.9	662.3	238.4
	N150	741.4	872.2	828.6	266.7
	N200	735.8	865.7	822.4	254.1
	Mean	634.9	728.2	697.1	231.7
CD @ 5%	C	4.9	21.9	15.5	5.7
	N	7.0	31.0	21.9	8.0
	C X N	9.8	43.8	30.9	11.3
Root iron (µg g^{-1} DW)					
HD 2967	N0	881.6	1102.1	936.7	638.6
	N120	1210.7	1267.4	1077.3	952.9
	N150	1332.1	1330.7	1131.1	1050.6
	N200	1322.1	1397.3	1187.7	1045.1
	Mean	1186.6	1274.4	1083.2	921.8
DPW-621-50	N0	917.7	1159.4	985.5	572.6
	N120	1273.7	1333.3	1133.3	854.4
	N150	1401.3	1399.9	1189.9	942.1
	N200	1404.1	1469.9	1261.3	937.0
	Mean	1249.2	1340.6	1142.5	826.5
CD @ 5%	C	9.8	13.7	11.7	8.8
	N	13.8	19.4	16.5	12.4
	C X N	19.6	27.4	23.3	17.6

Table 7: Effect of fertilizer nitrogen application on shoot and root zinc concentration at different growth stages in wheat

Wheat Cultivar (C)	Treatment (N)	Days After Sowing (DAS)			
		30	60	90	At Harvest
Shoot zinc (µg g^{-1} DW)					
HD 2967	N0	209.805	314.707	330.443	181.744
	N120	313.085	469.628	493.109	271.210
	N150	341.433	617.533	648.410	291.785
	N200	343.355	618.700	649.635	292.336
	Mean	301.920	505.142	530.399	259.268
DPW-621-50	N0	229.000	332.050	381.858	210.022
	N120	338.333	490.583	564.171	310.294
	N150	459.433	666.178	699.487	384.718
	N200	453.341	657.344	690.212	379.616
	Mean	370.027	536.539	583.932	321.162
CD @ 5%	C	2.347	3.852	4.240	2.218
	N	3.319	5.448	5.996	3.137
	C X N	4.694	7.704	8.480	4.436
Root zinc (µg g^{-1} DW)					
HD 2967	N0	608.781	639.220	543.337	516.170
	N120	845.812	888.102	754.887	641.654
	N150	846.420	888.741	755.430	642.115
	N200	842.963	885.111	752.344	639.493
	Mean	785.994	825.294	701.500	609.858
DPW-621-50	N0	607.458	722.665	543.337	461.837
	N120	718.940	1078.410	810.805	689.184
	N150	851.390	1189.010	893.960	759.866
	N200	846.853	1182.674	889.196	755.816
	Mean	756.160	1043.190	784.324	666.676
CD @ 5%	C	5.990	7.569	5.907	5.070
	N	8.471	10.704	8.354	7.170
	C X N	11.980	15.137	11.815	10.139

Effect of soil N supply on translocation index of Fe and Zn

Translocation index which is a measure of efficiency of nutrient translocation to the shoot in comparison to total uptake of the nutrient in question and the efficiency of translocation of mineral nutrients from the senescing shoot to the developing grains, did not vary significantly between the two bread wheat cultivars. N supply to the soil does significantly improve the root to shoot translocation of Fe and Zn (Table 8) at all stages of growth when compared with no N application treatment (N0). Root to shoot TI, irrespective of N treatment, or crop growth stage or cultivar, was higher for Zn than for iron. Mean translocation index, averaged over different N treatments, for the investigated micronutrients increased with crop growth upto 90DAS but declined thereafter at harvest. This is quite expected as shoot demand for nutrients grows until the plant reaches its peak vegetative growth and transition into the reproductive growth but declines as the leaf senescence sets in. Cereals being strategy II plants, Fe and Zn are presumably also translocated in the phloem as complexes with phytosiderophore (PS) (Curie *et al*., 2009) [11]. The Fe-PS complexes are mainly taken up by the plasma membrane-bound transporter YS1 (Curie *et al*., 2001; Schaaf *et al*., 2004) [12, 30]. Further these phytosiderophores are metallophores and YS1 or YS1-like (YSL) transporters are also thought to mediate Zn-PS uptake and that of other metal micronutrients (Schaaf *et al*., 2004) [30]. It is likely that high N supply increases the expression level of YS1 transporter which in turn may increase translocation of Fe and Zn from root to shoot in both the experimental wheat cultivars. Thus, this increase in translocation may be due to N mediated upregulation of Fe and Zn transporters like YS1 (yellow stripe 1) and also upregulation of enzymes involved in NA biosynthesis like Nicotianamine Synthase 1(NAS1). Role of NA/DAS in retranslocation of micronutrients is suggested (Shamima, 2016) [31].

Table 8: Effect of fertilizer nitrogen application on iron and zinc translocation index at different growth stages in wheat

Wheat Cultivar (C)	Treatment (N)	Days After Sowing (DAS)			
		30	60	90	At Harvest
Iron translocation index					
HD 2967	N0	0.321	0.308	0.332	0.267
	N120	0.333	0.359	0.385	0.262
	N150	0.336	0.373	0.400	0.301
	N200	0.336	0.360	0.386	0.282
	Mean	0.331	0.350	0.376	0.278
DPW-621-50	N0	0.314	0.301	0.325	0.227
	N120	0.333	0.336	0.369	0.218
	N150	0.346	0.384	0.410	0.221
	N200	0.344	0.371	0.395	0.213
	Mean	0.334	0.348	0.375	0.220
CD @ 5%	C	0.001	0.007	0.005	0.005
	N	0.001	0.010	0.008	0.007

	C X N	0.001	0.014	0.011	0.010
Zinc translocation index					
HD 2967	N0	0.256	0.330	0.378	0.260
	N120	0.270	0.346	0.395	0.297
	N150	0.287	0.410	0.462	0.312
	N200	0.289	0.411	0.463	0.314
	Mean	0.276	0.374	0.425	0.296
DPW-621-50	N0	0.274	0.315	0.413	0.313
	N120	0.320	0.313	0.410	0.310
	N150	0.350	0.359	0.439	0.336
	N200	0.349	0.357	0.437	0.334
	Mean	0.323	0.336	0.425	0.323
CD @ 5%	C	0.002	0.002	0.002	0.002
	N	0.002	0.003	0.003	0.003
	C X N	0.004	0.004	0.005	0.004

Effect of fertilizer nitrogen application on physiological nutrient use efficiency (NUE^{Fe}, NUE^{Zn} and NUE^{N})

Physiological NUE measured for the studied macro and micro nutrients i.e., N, Fe and Zn are presented in table 9. NUE^{N} was highest at N0 and declined with increasing N application for both the experimental wheat cultivars. Similar effect of N was measured on NUE^{Fe} and NUE^{Zn}, as highest values for nutrient use efficiency were recorded under N0 condition than with N application. Between the N doses, NUE^{Fe} and NUE^{Zn} increased with increasing N availability, however NUE^{N} declined with increasing soil N availability/ N application in a dose dependant manner. Between the two cultivars, cv DPW-650-21 showed significantly higher mean NUE^{Fe} than cv. HD-2967, while differences for NUE^{Zn} and NUE^{N} were not significant (Table 9). Reduction in nutrient use efficiency with N application may be a dilution effect as increasing in vegetative mass with N application is much more rapid than the parallel increase the micronutrient uptake. Role of micronutrient application for improving use efficiency of macronutrients was highlighted by Malakouti (2008). It was mooted to maintain sufficient rather than critical level of micronutrients in the soil to sustain quality grain production.

Table 9: Effect of fertilizer nitrogen application on physiological nutrient use efficiency for iron (NUE^{Fe}), zinc (NUE^{Zn}) and nitrogen (NUE^{N}) in wheat

Wheat Cultivar (C)	Treatment (N)	Physiological iron, zinc and nitrogen use efficiency		
		NUE^{Fe}	NUE^{Zn}	NUE^{N}
HD 2967	N0	0.440	0.707	0.215
	N120	0.322	0.583	0.190
	N150	0.279	0.571	0.164
	N200	0.324	0.645	0.187
	Mean	0.341	0.627	0.189
DPW-621-50	N0	0.551	0.799	0.241
	N120	0.357	0.516	0.190
	N150	0.372	0.520	0.183
	N200	0.383	0.532	0.187
	Mean	0.416	0.591	0.200
CD @ 5%	C	0.004	0.006	0.002
	N	0.005	0.009	0.003
	C X N	0.007	0.013	0.004

Relationship between plant N nutrition and grain micronutrients (Fe and Zn)

An insight into the correlation matrix structured to assess the relationship between different growth and nutritional attributes across N treatments clearly reveals a strong significant dependence of grain accumulation of Fe and Zn on activities of key enzymes of N metabolism (Table 10). A significant positive correlation was measured between grain Fe, Zn and grain N/ protein content. Root characteristics are stimulated under increasing N supply and that traits like root surface area correlated positively to the uptake and grain accumulation of Fe and Zn. High root surface area shall ensure greater uptake of micronutrients and their relatively improved partitioning to the shoot and to the grain. Flag leaf area showed high significant positive correlation with the activity of N assimilating enzymes and with grain Fe, Zn and N, indicating dual possibilities of influence i.e., firstly, it may garner more nutrients as a dominant sink and secondly, on senescence at maturity, may retranslocate more mineral nutrients to the grains when compared to plants supporting a smaller leaf area.

Table 10: Correlation Matrix for Flag leaf area, Root Surface Area (RSA), grain Fe, Grain Zn, Glutamine synthetase activity (GS), Nitrate reductase activity (NR), grain N and grain protein (All correlation values are significant at 1% level of probability)

Parameter	Flag leaf area	RSA	Grain Fe	Grain Zn	GS	NR	Grain N	Grain Protein
Flag leaf area	1	0.956	0.966	0.933	0.910	0.945	0.976	0.964
RSA		1	0.995	0.994	0.777	0.833	0.99	0.998
Grain Fe			1	0.979	0.820	0.867	0.996	0.997
Grain Zn				1	0.718	0.785	0.972	0.988
GS					1	0.983	0.844	0.801

NR						1	0.881	0.857
Grain N							1	0.991
Grain Protein								1

Conclusion

Improving the N fertilization levels appears to be an effective tool for the bio-fortification of wheat grains with iron (Fe) and zinc (Zn). Greater shoot to total translocation indices with increasing N fertilization levels suggests that optimizing N supply is critical to promote root to shoot translocation of Fe and Zn to enable shoots with high Fe and Zn accumulation. Optimal N management of crop shall not only ensure high micronutrient uptake and their partitioning to the shoot but shall also ensure high grain yield and high Fe and Zn concentration in the grain. Further, the positive influence of N on root Fe and Zn uptake, root to shoot Fe and Zn translocation, and accumulation in grain and shoot are of significance for Fe and Zn biofortification in cereal crops like wheat and rice. Increased concentration of grain micronutrients with N application may be caused by direct involvement of N in creating more demanding/powerful sinks (Kutman *et al*., 2011) [21] or could be related to N effect on synthesis of NA and/or DMA (Shamima 2016) [31] which are chelators of Fe and Zn and aid in latter's retranslocation from the developed or senescing leaf to the younger developing leaf/grain.

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