Site-specific factors influence the field performance of a Zn-biofortified wheat variety

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Abstract

Background: Biofortification of wheat with zinc (Zn) through breeding and agronomy can reduce Zn deficiencies and improve human health. 'High-Zn' wheat varieties have been released in India and Pakistan, where wheat is consumed widely as a dietary staple. The aim of this study was to quantify the potential contribution of a 'high-Zn' wheat variety (*Triticum aestivum* L. var. *Zincol-2016*) and Zn fertilisers to improving dietary Zn supply under field conditions in Pakistan.

Methods: Grain Zn concentration of *Zincol-2016* and local reference varieties were determined at three sites of contrasting soil Zn status: Faisalabad (Punjab Province; diethylenetriamine pentaacetate- (DTPA-)extractable Zn, 1.31 mg kg^{-1} soil; gross plot size 13.3 m^2; n=4; reference var. *Faisalabad-2008*), Islamabad (Capital Territory; 0.48 mg kg^{-1}; 4.6 m^2; n=5; reference var. *NARC-2011*), and Pir Sabak (Khyber Pakhtunkhwa, KPK, Province; 0.12 mg kg^{-1} soil; 9.1 m^2; n=4; reference vars. *Pirsabak-2015*, *Wadhan-2017*). Eight Zn fertiliser treatment levels were tested using a randomised complete block design: control; soil (5 or 10 kg ha^{-1} $ZnSO_4.H_2O$; 33% Zn applied at sowing); foliar (0.79 or 1.58 kg of $ZnSO_4.H_2O$ ha^{-1} applied as a 250 L ha^{-1} drench at crop booting stage); three soil × foliar combinations.

Results: At the Faisalabad site, the grain Zn concentration of *Zincol-2016* was greater than *Faisalabad-2008*, with no yield penalty. *Zincol-2016* did not have larger grain Zn concentrations than reference varieties used at Islamabad or Pir Sabak sites, which both had a lower soil Zn status than the Faisalabad site. Foliar Zn fertilisation increased grain Zn concentration of all varieties at all sites. There were no significant effects of soil Zn fertilisers, or variety·fertiliser interactions, on grain Zn concentration or yield.

Conclusions: Environment and management affect the performance of 'high-Zn' wheat varieties, and these factors needs to be evaluated at scale to assess the potential nutritional impact of Zn biofortified crops. Designing studies to detect realistic effect sizes for new varieties and crop management strategies is therefore an important consideration. The current study indicated that nine replicate plots would be needed to achieve 80% power to detect a 25% increase in grain Zn concentration.

Keywords

Biofortification, calcium (Ca), cadmium (Cd), environment, genotype, G×E×M, iron (Fe), management, selenium (Se), zinc (Zn)

Introduction

Zinc (Zn) is an essential micronutrient for all organisms (Broadley et al., 2007). Recommended dietary intake values vary depending on demographic and dietary factors, however, a weighted Estimated Average Requirement (EAR) of 10.3 mg d^{-1} has been estimated at a global scale (Kumssa et al., 2015). The EAR is the quantity of a nutrient required to meet the needs of half the individuals in an age- and sex-specific population group. For most individuals, the primary route of intake of Zn is from food sources. An estimated 17% of the global population is at risk of Zn deficiency due to inadequate supplies of Zn in national food systems (Wessells and Brown, 2012; Kumssa et al., 2015). The risk of Zn deficiency increases in areas where the consumption of animal source foods is limited, including many countries in South Asia and sub-Saharan Africa. Estimates of the prevalence of Zn deficiency from food supply are likely to be conservative, based on evidence from population-based surveys of biomarkers of Zn status (Zn concentration in blood plasma or serum) and the incidence of proxies of Zn deficiency including diarrhoea and stunting (low height for age in children), which indicate that Zn deficiency risks are larger (King et al., 2016).

Wheat (*Triticum aestivum* L.) is an important cereal crop and a major source of dietary Zn globally, especially in South Asia where risks of dietary Zn deficiency are likely to be large. For example, Akhtar (2013) found that the prevalence of Zn deficiency exceeded 40% among women and children in India and Pakistan, based on surveys of blood plasma/serum Zn status. In India, Zn concentration in wheat grain, among a panel of 36 diverse genotypes grown in experimental plots on contrasting soil types, ranged from 24.9–34.8 mg kg^{-1} (Khokhar et al., 2017, 2018). In Pakistan, the concentration of Zn in wheat grain collected from farmers' fields in 75 locations ranged from 15.1–39.7 mg kg^{-1} (Joy et al., 2017). Among a panel of 28 wheat genotypes of Pakistani origin, grown over two seasons at a single location, grain Zn concentration ranged from 21.2–33.3 mg kg^{-1} with a mean of 27.5 mg kg^{-1} (Rehman et al., 2018b). Assuming a whole-grain Zn concentration of 30 mg kg^{-1}, an energy density for wheat grain of 3400 kcal kg^{-1}, and a dietary wheat supply of 517 and 903 kcal capita d^{-1} in India and Pakistan, respectively (FAOSTAT, 2020), the supply of Zn from whole-grain wheat represents 4.6 and 8.0 mg $capita^{-1}$ d^{-1}, i.e. 45% and 78% of the weighted EARs, for India and Pakistan respectively.

The HarvestPlus programme and their partners have used conventional breeding to develop and release new 'high-Zn' wheat varieties in India and Pakistan, a process known as genetic biofortification (Velu et al., 2015; Singh and Velu, 2017). These new varieties have been developed from synthetic wheat lines derived from wild wheat relatives, including *Aegilops tauschii* (D genome donor of wheat), *Triticum spelta* and wild *T. dicoccon*, and crosses with *T. durum*. The HarvestPlus target was to enhance the Zn concentration in grain of existing wheat varieties by 8–12 mg kg^{-1}, above a notional baseline whole-grain Zn concentration of 25 mg kg^{-1}, without reducing yield or quality (Velu et al., 2015). In India, 'high-Zn' varieties have been developed and released in the North Eastern Plain Zone (NEPZ): *Abhay* (*Zinc Shakthi*, *Chitra*), *Akshai* (*BHU-3*) and *BHU-6*, and in the North Western Plain Zone (NWPZ): *WB02* and *HPBW-01* (Velu et al., 2015; Singh and Velu, 2017). In Pakistan, a 'high-Zn' wheat variety *Zincol-2016*, developed by National Agriculture Research System (NARS) from a background *NARC-2011* variety, was released by the Pakistan Agriculture Research Council (PARC) in 2016.

In addition to genetic approaches, grain Zn concentration in wheat can also be increased with Zn-containing fertilisers, a process termed agronomic biofortification or agro-fortification (Cakmak, 2008; White and Broadley, 2009; Zhao et al., 2019). In a review of nine published field studies, Joy et al. (2015b) noted that foliar Zn ($ZnSO_4$) fertilisers, applied as a drench to field-grown wheat, increased the whole-grain Zn concentration by a median of 63%. Soil-applied Zn fertilisers can also increase grain Zn concentrations, albeit to a much lesser extent than foliar-applied Zn fertilisers but may also increase crop yield in some settings (Cakmak 2008; Zou et al., 2012). In a review of 14 published field studies, soil-applied Zn fertilisers increased whole-grain Zn concentration of field-grown wheat by a median of 19% (Joy et al., 2015b). In Pakistan, soil-applied Zn fertilisers led to an increase in the Zn concentration of whole-grain *chapati* flatbread, from 18±2 to 24±2 mg kg^{-1} (mean±SD) (Ahsin et al., 2019). In India, wheat agro-fortified with foliar Zn fertiliser and supplied as a Zn-enriched flour for six months to women and children aged from 4 to 6 years resulted in a 17% and 40% reduction in self-reported incidences of pneumonia and vomiting, respectively (Sazawal et al., 2018).

There is a lack of information in the literature on how new HarvestPlus wheat varieties perform under field conditions in India and Pakistan compared to widely-grown varieties. However, there is evidence from pot studies that there are likely to be strong genotype (G) × environment (E) × management (M) effects on grain Zn concentration. In a recent pot-study, using an alkaline calcareous soil with a small concentration of plant-available Zn (0.7 mg kg^{-1})

diethylenetriamine pentaacetate- (DTPA-) extractable Zn, Hussain et al. (2018) reported that *Zincol-2016* (~22 mg kg^{-1}) had a larger grain Zn concentration than *Faisalabad-2008* (~18 mg kg^{-1}). When Zn fertiliser was added to soils, the differences in grain Zn between *Zincol-2016* (~36 mg kg^{-1}) and *Faisalabad-2008* (~25 mg kg^{-1}) increased markedly. In a pot study by Yousaf et al. (2019), *Zincol-2016* (33.9 mg kg^{-1}) had a much larger grain Zn concentration than *Faisalabad-2008* (23.8 mg kg^{-1}) in unfertilised soils. However, genotypic differences were not evident when foliar or soil Zn fertilisers were added and which increased the grain Zn concentration in both varieties. In a pot study by Yaseen and Hussain (2020), *Zincol-2016* had a greater grain Zn concentration than a reference variety, *Jauhar-2016,* when Zn fertilisers were added to alkaline calcareous soils although there was no genotypic difference in grain Zn concentration under control conditions. The aim of this study was to quantify the potential contribution of *Zincol-2016* to improving the dietary supply of Zn under experimental field conditions. Field experiments were established in Pakistan at three sites of contrasting soil Zn status, where *Zincol-2016* was grown in replicated plots and compared with local reference lines, with and without soil and/or foliar Zn fertilisers.

Materials and Methods

Site selection and characterisation

Experiments were established at three sites of contrasting Zn status. The site at Faisalabad had a high DTPA-extractable Zn concentration, whereas the sites at Islamabad and Pir Sabak had medium and low DTPA-extractable Zn concentration, respectively. A DTPA-extractable soil Zn concentration of 0.8–1.0 mg kg^{-1} is considered adequate for the growth of most crops (Lindsay and Norvell, 1978). Soils at all three sites had high pH, which is typical of calcareous soils in the region. Properties of the soil at the three locations are given in Table 1.

Experimental design and layout

The experiments sought to test the effect of variety and Zn fertilisers on wheat grain yields and Zn concentration at each of the three sites. The choice of variety was site-specific, so that the performance of *Zincol-2016* could be compared directly with reference varieties used routinely by farmers in the same locations (Table 1). At all sites, eight Zn fertiliser treatment levels were tested (Table 2): control; soil-applied (5 or 10 kg ha^{-1} $ZnSO_4.H_2O$; 33% Zn applied at sowing); foliar-applied (0.79 or 1.58 kg of $ZnSO_4.H_2O$ ha^{-1} applied as a 250 L ha^{-1} drench at crop booting stage, Zadoks' scale 45-50; Zadoks et al., 1974); and three combinations of soil- and foliar-applied $ZnSO_4.H_2O$ comprising 5+0.79 kg ha^{-1} soil+foliar, 10+0.79 kg ha^{-1} soil+foliar, and 10+1.58 kg ha^{-1} soil+foliar. A complete randomised block design was adopted at each site, comprising four replicates at Faisalabad and Pir Sabak, and five replicates at Islamabad. Layout details are provided in Supplementary Information.

Agronomy

The gross plot sizes were: Faisalabad 13.3 m^2 (3.35 × 3.96 m), Islamabad 4.6 m^2 (1.52 × 3.05 m), and Pir Sabak 9.1 m^2 (2.13 × 4.27 m). Soil was ploughed three times then levelled by planking. Plot boundaries were marked manually at all the sites. Seed of the selected varieties (Table 1) were sown using a seed rate of 125 kg $ha^{–1}$ using row spacing of ~25 cm. The crop was sown on 24 November 2018 at Pir Sabak, 02 December 2018 at Islamabad, and 08 December 2018 at Faisalabad. A total of five irrigations were made during crop growth at Pir Sabak and Faisalabad, with three irrigations at Islamabad which received greater rainfall.

General fertiliser applications comprised basal phosphorus (di-ammonium phosphate, P_2O_5 46%) at 115 kg P_2O_5 ha^{-1}, and potassium (muriate of potash, K_2O 60%) at 75 kg K_2O ha^{-1} at Faisalabad and Pir Sabak. Potassium was not applied at Islamabad as soil testing indicated adequate potassium status. Basal fertilisers were applied at time of soil preparation, prior to sowing. Nitrogen (urea at 110 kg ha^{-1}) was split in to two halves, one half-applied at time of first irrigation (Zadoks' scale ~25) whereas the remaining half at Zadoks' scale ~40). Soil-applied Zn fertiliser was broadcast uniformly in the designated treatment plot(s) and incorporated into the soil before sowing. The foliar treatment for Zn fertilisers was applied in the early morning hours to reduce risk of leaf-scorch.

Measurements of yield and yield components

Prior to harvest (May 2019), crop measurements were taken at five random locations within the plot to exclude border effects. These included plant height, number of tillers per square meter, spike length, number of grains in 10 spikes, grain weight for 10 spikes, and crop biomass. After on-site harvest/threshing of whole treatment plot, wheat grain yield was determined for each treatment and then converted into kg ha^{-1}. A 500 g subsample was taken out of well-mixed threshed grain from each treatment plot, out of which 50 g was preserved for the analysis of grain Zn and other elemental concentrations.

Determining grain concentration of Zn and other elements

Grain digestion and elemental analysis methods are described in Khokhar et al. (2018, 2020). Briefly, approximately 10 grains (whole-grain) were dried, weighed, and soaked in 3 mL 70% Trace Analysis Grade (TAG) HNO_3 and 2 mL H_2O_2, at room temperature overnight, in perfluoroalkoxy (PFA) tubes (Anton Paar GmbH, Graz, Austria). The tubes were then placed into polyethylethylketone (PEEK) pressure jackets and digested in a Multiwave 3000 microwave system with a 48-vessel MF50 rotor (Anton Paar Gmbh). Whole-grain Zn concentration was determined by inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany). The Zn recovery from nine samples of a Certified Reference Material (CRM; Wheat flour SRM 1567b, NIST, Gaithersburg, MD, US; 11.61 mg kg^{-1}) was 94.4% (first run) and 91.2% (second run). The Limit of Detection (LOD) for Zn, equivalent to 3 times the standard deviation (SD) of the concentrations of all of the operational blanks and a notional dry weight of 0.35 g was 4.45 and 2.47 mg kg^{-1} for the first and second analysis runs, respectively. The full range of elements reported from the ICP-MS were Ag, Al, As, B, Ba, Be, Ca, Cd, Cr, Co, Cs, Cu, Fe K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, Tl, U, V, and Zn (Supplementary Information). Data for Zn, Fe, Cd and Ca are reported here.

Data analyses

All statistical analyses were conducted on the R platform (R Core Team, 2017). First, analysis of variance (ANOVA) was used to test the main effects of variety, fertiliser treatments, and their interaction. Exploratory plots (histograms and QQ plots of the residuals for the analysis) were then examined to check the plausibility of the assumption that these are drawn from a normal distribution, and the plot of residuals against fitted values was examined to check the plausibility that the variance of the residuals was homogeneous. At this point a decision would be made to transform the data to make these assumptions plausible, although that was not needed for the analyses reported in this study.

If the main effect of fertiliser appeared significant, then it was examined further by testing a set of contrasts among levels of the fertiliser factor against the Residual Mean Square (RMS) for the overall ANOVA. The treatments used in the study do not naturally partition into a set of informative orthogonal contrasts. Therefore, we examined a set of non-orthogonal contrasts, controlling the family-wise error rate with Holm's modification of Bonferroni's method (Holm, 1979), and we reported adjusted p-values. Sokal and Rohlf (2012) recommend this approach when examining non-orthogonal contrasts. Given that power is lost for each additional test, four informative contrasts were selected (Table 2) and the treatment by variety interaction was not partitioned for the contrast analyses. Effect sizes for all four contrasts, and a (pooled) standard error are reported. R scripts are provided in Supplementary Information).

The contrasts were defined before any data from the experiment were examined. The rationale for this choice of contrasts was to explore the largest respective effects of soil application and foliar application (C1 and C2) relative to the no-fertiliser control, and then to examine the evidence for an incremental improvement from a large-rate soil application when a single foliar application is in use (C3, "with a standard foliar application, is there any benefit in applying Zn to the soil as well?"), and from adding a double foliar application when a large-rate soil application is in use (C4, "when applying Zn to the soil, is there a supplementary benefit of applying a foliar dose as well?"). As is noted above, these 4 contrasts, each with 1 degree of freedom, are not orthogonal. That is to say the contrasts are not independent of each other, and so do not give independent tests on components of the sum of squares for treatments.

Results

The outputs of the ANOVA for treatment factors, their interactions, and selected contrasts, for the variates of yield and grain Zn, Fe, Ca and Cd concentration are presented in Table 3. Arithmetic means across the plots for these same variates are plotted in Figure 1; individual plot-level data, including yield components, are provided as Supplementary Information. Fertiliser treatment means, and the effects sizes of the chosen contrasts, are presented in Tables 4 and 5, respectively. The interpretation of the effects sizes is conditional on the signs (i.e. a positive value for C1 would indicate that the mean for the soil Zn treatment is larger than the mean for the control). The standard error is obtained from the pooled RMS, so it is the same where replication sizes are equal.

Grain yield

At all three sites, there was no evidence to reject the null hypothesis of no effect of Zn fertiliser application, or variety·Zn fertiliser interaction, on yield (Table 3). The lack of yield responses to Zn fertilisers was unexpected given that wheat is generally responsive to Zn fertilisers on calcareous soil types in Pakistan (e.g. Joy et al., 2017; Rehman et al., 2018a; Asif et al., 2019). At the Faisalabad and Islamabad sites, there was no evidence to reject the null hypothesis of no difference in mean yield among varieties, however, there was some evidence to reject this null hypothesis at the Pir Sabak site (p=0.024; Table 3), with *Wadhan-2017* having a slightly greater yield than *Pirsabak-2015* and *Zincol-2016*. The overall grain yield of *Zincol-2016* and *Faisalabad-2008* was ~50% of those observed for *Zincol-2016* and reference varieties at Islamabad and Pir Sabak. The soil texture at the Faisalabad site is "sandy loam" where one would always expect a yield penalty compared to the "silt loam" textured soils at the other locations. There was also a yellow rust attack at the time of grain formation/development at the Faisalabad site and surrounding area in 2019.

Grain zinc concentration

There was strong evidence to reject the null hypothesis of no difference in grain Zn concentration between the varieties at the Faisalabad (p<0.001) and Pir Sabak (p=0.002) sites, (Table 3). At Faisalabad, *Zincol-2016* had a consistently larger grain Zn concentration than *Faisalabad-2008*; a difference of ~16% averaged across all 8 fertiliser treatment levels (Figure 1; Supplementary Information). At Pir Sabak, grain Zn concentration decreased in the order *Wadhan-2017* > *Zincol-2016* > *Pirsabak-2015*. At Islamabad, there was no evidence to reject the null hypothesis of no difference in grain Zn concentration between the varieties (p=0.186; Table 3).

There was evidence to reject the null hypothesis of no effect of Zn fertiliser application on grain Zn concentration at all three sites (Table 3): Faisalabad (p=0.028), Islamabad (p=<0.001), and Pir Sabak (p=0.002). Application of foliar Zn fertiliser increased grain Zn concentration at all three sites (Tables 3-5). Thus, at Faisalabad, foliar Zn fertiliser application increased grain Zn concentration by 6.9 (Contrast 2, C2) and 7.1 (C4) mg kg^{-1}. At Islamabad, foliar Zn fertiliser application increased grain Zn concentration by 18.0 (C2) and 19.1 (C4) mg kg^{-1}. At Pir Sabak, foliar Zn fertiliser application increased grain Zn concentration by 10.4 (C2) and 10.0 (C4) mg kg^{-1}. There was no evidence of any significant effect of soil Zn fertiliser application on grain Zn concentration at any of the sites based on the analyses of C1 or C3 contrasts (Table 3). There was no evidence of variety·Zn fertiliser interactions on grain Zn concentration at any of the three sites (Table 3).

Grain iron concentration

There was evidence to reject the null hypothesis of no difference among the varieties with respect to grain Fe concentration at the Faisalabad (p=0.011) and Islamabad (p=0.024) sites. At Faisalabad, *Zincol-2016* had a larger grain Fe concentration than *Faisalabad-2008*; a difference of ~12% averaged across all 8 fertiliser treatment levels (Figure 1; Supplementary Information). At Islamabad, *Zincol-2016* had a larger grain Fe concentration than *NARC-2011*; a difference of ~6% averaged across all 8 fertiliser treatment levels (Figure 1; Supplementary Information). At the Pir Sabak site, the null hypothesis of no difference among the varieties with respect to grain Fe concentration was retained (p=0.212; Table 3).

There was no evidence to reject the null hypothesis of no effect of Zn fertiliser application on grain Fe concentration at Faisalabad (p=0.995) or Pir Sabak (p=0.540) sites (Table 3). However, there was evidence to reject this null hypothesis at the Islamabad site (p<0.001; Table 3), with the contrasts effect sizes being 5.1 (C2) and 8.5 (C4) mg kg^{-1}. There was no evidence of any significant effect of soil Zn fertiliser application on grain Fe concentration, at Islamabad or the other two sites based on the analyses of C1 or C3 contrast (Table 3). There was no evidence of variety·Zn fertiliser interactions on grain Fe concentration at any of the three sites (Table 3).

Grain calcium concentration

There was evidence to reject the null hypothesis of no difference among the varieties with respect to grain Ca concentration at the Faisalabad (p<0.001) and Pir Sabak (p<0.001) sites, (Table 3). At Faisalabad, *Faisalabad-2008* had a larger grain Ca concentration than *Zincol-2016*; a difference of ~68% averaged across all 8 fertiliser treatment levels (Figure 1; Supplementary Information). At Pir Sabak, *Zincol-2016* had a larger grain Ca concentration than *Wadhan-2017*; a difference of ~20% averaged across all 8 fertiliser treatment levels (Figure 1; Supplementary Information). However, at Pir Sabak, *Pirsabak-2015* had a larger grain Ca concentration than *Zincol-2016*; a difference of ~5% averaged across all 8 fertiliser treatment levels. At the Islamabad site, there was no evidence for varietal differences in grain Ca concentration (p=0.582; Table 3). There was no evidence of any effects of Zn fertiliser, or variety·Zn fertiliser interactions, on grain Ca concentration at any of the three sites (Table 3).

Grain cadmium concentration

There was evidence to reject the null hypothesis of no difference among the varieties with respect to grain Cd concentration at the Pir Sabak site (p<0.001), (Table 3). *Zincol-2016* had a larger grain Cd concentration than *Pirsabak-2015*; a difference of ~34% averaged across all fertiliser treatments. However, *Wadhan-2017* had a larger grain Cd concentration than *Zincol-2016*; also a difference of ~34% averaged across all 8 fertiliser treatment levels (Figure 1;

Supplementary Information). There was no evidence for varietal differences in grain Cd concentration at the Faisalabad ($p=0.055$) and Islamabad ($p=0.805$) sites (Table 3). The null hypothesis of no effect of Zn fertiliser application on grain Cd concentration was retained at Faisalabad ($p=0.660$) and Islamabad ($p=0.716$) sites; there was weak evidence to reject this null hypothesis at the Pir Sabak site, ($p=0.035$; Table 3), with an effect size of -0.005 mg kg^{-1} in contrast C2 (Tables 4, 5). There was no evidence of variety·Zn fertiliser interactions on grain Cd concentration at any of the three sites (Table 3).

Discussion

The primary focus of this study was to determine the effects of growing location and Zn fertilisers on the grain Zn concentration of a variety of biofortified wheat, *Zincol-2016*, compared to local elite reference varieties. Experiments were conducted at three sites of contrasting soil Zn status in Pakistan. In the absence of Zn fertilisers, the grain Zn concentration of *Zincol-2016* was greater than the local variety at only one of the sites, Faisalabad. At the other two sites, Islamabad and Pir Sabak, *Zincol-2016* did not have a greater grain Zn concentration than the local varieties. Grain yields were markedly lower at Faisalabad than Islamabad and Pir Sabak, however, there was no evidence for differences in yield between the varieties at the Faisalabad site. Conversely, there were yield differences between the varieties at the Islamabad site, but no evidence for differences in grain Zn concentration between the varieties. These observations indicate that variation in grain Zn concentration is not simply reflecting a yield dilution effect.

The experiments reported in this current study were not designed to test for effects of site on varietal performance. However, it is noteworthy that soils at Faisalabad had a larger concentration of DTPA-extractable soil Zn than the soils at the other two sites. Several studies have reported significant positive correlations between DTPA-extractable soil Zn concentration and wheat grain Zn concentrations under field conditions. For example, in a recent study in China, wheat grain Zn concentration correlated positively with soil available Zn in single wheat, wheat-maize, and rice-wheat cropping systems (Huang et al., 2019). Similar positive correlations have also been reported under field conditions in Iran (Karami et al., 2009), France (Oury et al., 2006), and Slovakia (Krauss et al., 2002). However, whilst available soil Zn clearly has predictive power, wheat grain Zn concentration is a complex trait which is influenced by many additional soil, varietal, and climatic factors (Karami et al., 2009; Huang et al., 2019).

Foliar Zn fertilisation increased the grain Zn concentration of all varieties at all sites. This observation is consistent with a large body of evidence that foliar Zn fertilisers are an effective method to increase the grain Zn concentration of field-grown wheat and other crops, and in many countries (Zou et al., 2012; Joy et al., 2015b; Ram et al., 2016). The largest increase in grain Zn concentration in the current study, as a result of foliar Zn fertilisers, was a 49% increase at the Islamabad site. Despite their potential effectiveness, including in studies from which self-reported health benefits have been noted (Sazawal et al., 2018), the use of foliar Zn fertilisers to enrich wheat grain is yet to be widely adopted by wheat growers in subsistence or commercial settings.

There were no significant effects of soil Zn fertilisers, or variety·fertiliser interactions, on grain Zn concentration at any of the sites. The use of soil Zn fertilisers has been reported to increase wheat grain Zn concentration in other field studies, albeit to a smaller extent than foliar Zn fertilisers (Joy et al., 2015b). For example, an average increase in grain Zn concentration of 12% was reported across 23 site-year combinations, spanning seven countries (Zou et al., 2012). Soil Zn fertilisers have also been reported to increase available Zn, for example, in a field study in Punjab Province, Pakistan, Ahsin et al. (2019) reported greater soil concentrations of DTPA-extractable Zn (1.1±0.1 mg kg^{-1}; mean±standard deviation, SD) in soils treated with Zn, than when no Zn fertilisers were applied (0.8±0.1 mg kg^{-1}). Soil applications of Zn fertilisers have specifically been shown to be effective at increasing the grain Zn concentration of *Zincol-2016* in pot experiments (Yousaf et al., 2019; Yaseen and Hussain, 2020). However, further research is needed to understand the potential value of longer-term soil fertility building with soil Zn fertilisers with new Zn-biofortified wheat varieties under field conditions, including the potential for multi-year effects, and the use of other nutrients to augment Zn uptake and translocation to grain. For example, farmer management such as an increased use of nitrogen fertilisers (Xue et al., 2012) and organic inputs (Wood et al., 2018) can increase wheat grain Zn concentration in field settings. Similarly, an increased use of organic materials (Manzeke et al, 2019) and nitrogen fertilisers (Manzeke et al., 2014; 2020) has been reported to increase grain Zn concentration in field-grown maize in smallholder farming systems.

It is important to understand how new varieties of biofortified wheat perform on different soils and under different farm-management practices. This will enable the potential impact of biofortified wheat to be evaluated in terms of dietary Zn intake and thereby improve estimates of their effectiveness beyond farmer adoption rates (e.g. Joy et al., 2017). Dietary Zn intake is itself a key indicator for assessing population Zn status (King et al., 2016). There are advantages to using dietary intake indicators due to the inherent challenges in interpreting biochemical biomarkers of Zn status in humans. For example, decreases in plasma or serum Zn concentration arise due to inflammation (Likoswe et al., 2020; McDonald et al., 2020). Furthermore, health and development outcomes linked to Zn deficiency, such as pneumonia, diarrhoea, and stunting, have complex aetiologies beyond Zn status (King et al., 2016).

Dietary Zn intake will be affected by variation in wheat grain Zn concentration arising due to genotype, environment, and management (G × E × M). Large ranges of wheat grain Zn concentration, from 14–59 mg kg^{-1} were reported from a survey of 599 locations in China (Huang et al., 2019), and from 15.1–39.7 mg kg^{-1} in a survey of 75 farmers' fields in Pakistan (Joy et al., 2017). However, despite the considerable nutritional significance of this variation with respect to population-level dietary requirements for Zn, especially in countries where wheat is consumed in large quantities, the contribution of different components of G × E × M to variation in grain Zn concentration remains poorly understood.

In terms of dietary Zn intake, even small changes in Zn concentration in staple foods can translate into large effects on estimates of population-level prevalence of Zn deficiency. In the current study, an increase in grain Zn concentration of 1 mg kg^{-1} would increase dietary Zn intake by 0.27 mg $capita^{-1}$ d^{-1}, assuming a current dietary intake of Zn from wheat of 8 mg $capita^{-1}$ d^{-1} arising from a grain consumption of 266 g $capita^{-1}$ d^{-1} in Pakistan. An increase in grain Zn concentration of 4 mg kg^{-1} would increase dietary intakes by an average of >1 mg $capita^{-1}$ d^{-1} which is >10% of the EAR for Zn of ~10.3 mg $capita^{-1}$ d^{-1} in Pakistan (Kumssa et al., 2015). There is therefore clear scope for the agriculture sector to mitigate a projected 9% decrease in wheat grain Zn concentration arising due to greater atmospheric CO_2 (mid-21st Century scenario of 550 ppm; Smith and Myers, 2018). Intriguingly, a ~30% larger maize grain Zn concentration attributed to a particular Vertisol soil type in Malawi (Chilimba et al., 2011; Joy et al., 2015a), corresponded with a larger inherent dietary Zn intake of 1.6 mg $capita^{-1}$ d^{-1} based on composite dietary analyses among smallholder farming communities (Siyame et al., 2013). However, it was not possible to link this elevated Zn intake among farmers growing crops on the Vertisols to differences in Zn status based on biomarkers, likely because Zn concentrations in blood plasma/serum are under tight homeostatic control. Similarly, Sazawal et al. (2018) did not observe a change in biomarkers of Zn status among individuals consuming wheat grain with a 50% greater Zn concentration, following foliar Zn fertiliser application, although self-reported health improvements were noted over their six-month study period. These studies highlight the need to consider dietary Zn intake as part of decision support for managing Zn deficiency.

Given the importance of understanding (potentially subtle) effects of G $\times$ E $\times$ M contributions to grain Zn concentrations, to thereby enable accurate estimates of potential improvements to dietary Zn intake, it is critical that experiments and field surveillance activities are designed appropriately. In the current study, grain Zn concentration at the Islamabad site had a control treatment mean of 36.9 mg kg^{-1} and a residual mean square of 35.1 based on the overall ANOVA. A power analysis for an effect size of 50%, 33% or 25% in a simple control/treatment experiment is shown in Figure 2. This was done with the `Fpower` function from the `daewr` package for the R platform (Lawson, 2014). For a 25% effect size (i.e. an increase in grain Zn concentration of 9.2 mg kg^{-1}, from 36.9 to 46.1 mg kg^{-1}), nine or more replicates would be required to achieve 80% experimental power. The replication in the current study (n=5) is powered sufficiently to detect an effect size smaller than 50% but larger than 33%. Therefore, the power to detect subtle treatment effects in this study is small compared to the potential dietary importance of these effects.

Beyond Zn, wheat is an important dietary source of a range of other mineral micronutrients. Positive correlations between grain Zn and Fe concentrations have been reported when different varieties of wheat are being phenotyped (e.g. Khokhar et al., 2020). Interventions to increase dietary Zn intake through breeding might therefore have added nutritional benefits. For Fe, *Zincol-2016* had a larger grain Fe concentration than the local varieties at two of the three sites, Faisalabad (*cf. Faisalabad-2008*) and Islamabad (*cf. NARC-2011*), but not at Pir Sabak. For Ca, another important human micronutrient, *Zincol-2016* had a larger grain Ca concentration than *Faisalabad-2008* and *Wadhan-2017*, at Faisalabad and Pir Sabak, respectively. In contrast, *Zincol-2016* had a smaller grain Ca concentration than *Pirsabak-2015* at the Pir Sabak site. Whilst there was limited evidence that Zn fertiliser applications affected grain Fe (or Ca) concentrations, the site-specific varietal responses reported in this study show the importance of phenotyping grain for multiple nutrient elements during biofortification breeding programmes.

The grain concentrations of 19 mineral elements are reported in this current study (Supplementary Information). Beyond the traits of grain Zn, Fe, and Ca concentration, which are heritable and amenable to crop breeding (Khokhar et al., 2018), the grain concentration of other essential dietary micronutrients, such as selenium (Se), have low heritability and are influenced to a far greater extent by the soil environment in which the crop is grown (White and Broadley, 2009). Interestingly, grain Se concentration across all plots at Faisalabad (median 0.082 mg kg^{-1}; range 0.060–0.119) was almost five-fold greater than at Pir Sabak (median 0.017 mg kg^{-1}; range 0.008–0.033), dwarfing any potential effect of variety or agronomy in the current study. It will be interesting to discover if further evidence emerges of systematic – and nutritionally important – spatial variation in grain Se concentration across the major wheat growing areas of Pakistan, as has been observed in sub-Saharan Africa for wheat and *teff* (*Eragrostis tef* (Zucc.) Trotter; Gashu et al., 2020), and also for maize (Ligowe et al., 2020).

Beyond elements of nutritional value, it is also important to consider how G × E × M factors might affect the concentrations of potentially toxic elements in wheat grain. For example, *Zincol-2016* accumulated more Cd when grown in heavily contaminated soils in pots (Qaswar et al., 2017). In the current study, there was no evidence that *Zincol-2016* systematically accumulated more Cd in its grain than local varieties. At Faisalabad or Islamabad, there were no significant varietal differences in grain Cd concentration. Significant varietal differences in grain Cd concentration were observed at Pir Sabak, however, *Zincol-2016* had an intermediate grain Cd concentration compared to the two local varieties. The median grain Cd concentrations at all three sites (Faisalabad, 0.008 mg kg^{-1}; Islamabad, 0.027 mg kg^{-1}; Pir Sabak, 0.018 mg kg^{-1}) were below the maximum permissible grain Cd concentration of 0.1 mg kg^{-1} (WHO/FAO, 2016).

In addition to potentially toxic elements, it will also be important to determine how G × E × M factors will influence the concentration of phytate and other anti-nutritional factors which can inhibit the bioavailability of Zn, Fe, and other mineral nutrients in the human gut. Anti-nutritional factors were not considered in the current study. Interestingly, in the recent study of Yaseen and Hussain (2020), using alkaline calcareous soils, there were no genotypic differences in grain Zn or phytate concentration under control conditions between *Zincol-2016* and the reference variety *Jauhar-2016*. However, *Zincol-2016* had a greater grain Zn concentration and a lower phytate concentration than *Jauhar-2016* when Zn fertilisers were added, indicating that the bioavailable Zn would be greater in *Zincol-2016*.

Conclusions

Zincol-2016 is a new variety of wheat which has been released in Pakistan, having been bred to have a greater concentration of Zn in its grain. In field experiments conducted at three sites, the grain Zn concentration of *Zincol-2016* was greater than the local variety at just one of the sites. Varieties responded similarly to Zn fertilisers, with substantial increases in grain Zn concentration when foliar Zn fertilisers were applied. Soil Zn fertilisers had no significant effect on grain Zn concentration in this study. When evaluating the potential nutritional impact of biofortified crops it is important to understand how varietal performance is influenced by environmental and management factors, including soil type and crop management. Experiments and surveys should be powered appropriately for both target (in this case Zn) and non-target nutrient quality traits.

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Author contribution statement

MHZ, RML, and MRB designed the study. MHZ, IA, EHB, SDY, and LW conducted the field experiments and sample analyses. MHZ, RML, and MRB analysed the data. MHZ, RML, EJMJ, and MRB wrote the manuscript. All authors edited and approved the final version of the manuscript. MHZ, NML (BiZiFED Principal Investigator), MZ, and MRB secured funding for the research, with the support of the other co-authors.

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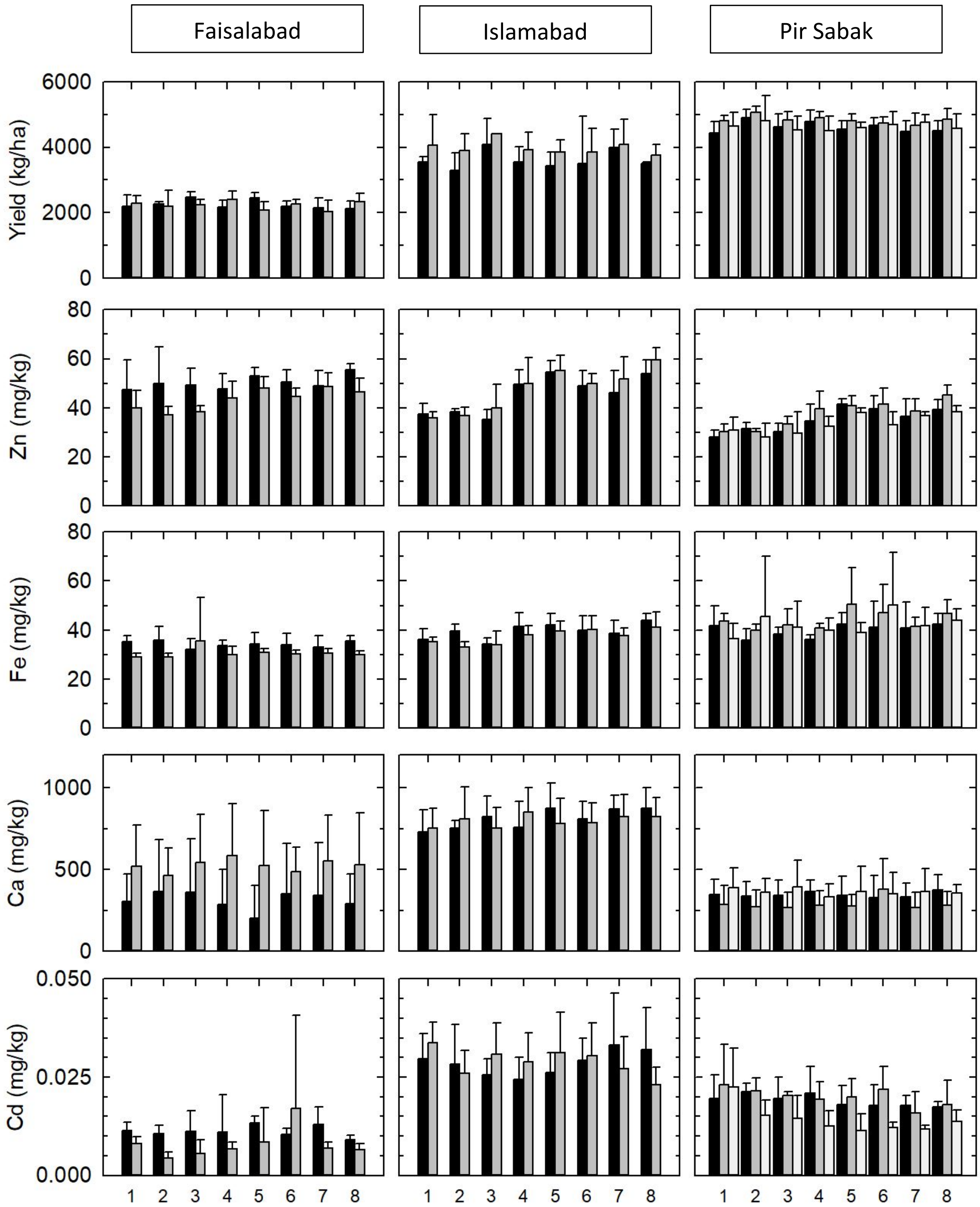

Figure 1. Arithmetic means (± standard deviation, SD) of grain yield and mineral concentration of wheat at three sites under control (Treatment 1, T1) or Zn-fertilised conditions (all units expressed as kg ha^{-1} $ZnSO_4.H_2O$: T2=5 soil; T3=10 soil; T4=0.79 foliar; T5=1.58 foliar; T6=5 soil and 0.79 foliar; T7=10 soil and 0.79 foliar; T8=10 soil and 1.58 foliar). Black bars are *Zincol-2016*; grey bars are local reference varieties (*Faisalabad-2008* at Faisalabad; *NARC-2011* at Islamabad; *Wadhan-2017* and *Pirsabak-2015* – lighter grey – at Pir Sabak).

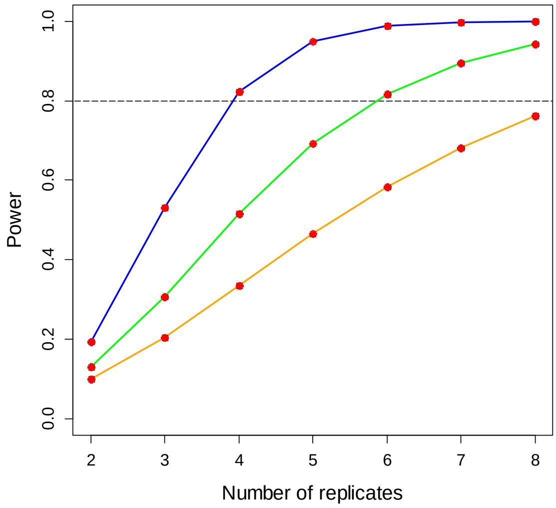

Figure 2. Power analysis for a simple control/treatment experiment for an effect size of 50% (blue line), 33% (green line) or 25% (orange line). Data are based on a treatment mean grain Zn concentration of 36.9 mg kg^{-1} and a residual mean square of 35.1, as observed at the Islamabad site.

Table 1. Locations (latitude, longitude), soil properties (median +/- standard deviation), and cultivars of wheat.

Location	Texture	pH[1]	Organic matter (%)[2]	DTPA-Zn (mg kg^{-1})[3]	Varieties
Faisalabad, Punjab 31.562619, 73.114814	Sandy loam	7.90±0.06	0.56±0.16	1.31±0.11	*Zincol-2016, Faisalabad-2008*
Islamabad, ICT 33.672367, 73.130277	Silt loam	8.35±0.06	0.77±0.10	0.47±0.03	*Zincol-2016, NARC-2011*
Pir Sabak, KPK 34.017751, 72.044491	Silt loam	8.30±0.04	0.97±0.07	0.11±0.06	*Zincol-2016, Pirsabak-2015, Wadhan-2017*

[1]Soil $pH_{1:2.5}$ (soil:water, NF X31-103 1988; AFNOR, 1994)
[2]Walkley (1947)
[3]Lindsay and Norvell (1978)

Table 2. Contrasts tested in this study four contrasts (C1–C4) represent non-orthogonal components of the fertiliser effect. Treatment 1 (T1) represents control conditions with no Zn fertilisers; T2-8 represent Zn-fertilised conditions (all units expressed as kg ha^{-1} $ZnSO_4.H_2O$: T2=5 soil; T3=10 soil; T4=0.79 foliar; T5=1.58 foliar; T6=5 soil and 0.79 foliar; T7=10 soil and 0.79 foliar; T8=10 soil and 1.58 foliar).

	Contrast			
Treatment	**C1**	**C2**	**C3**	**C4**
1	–1	–1	0	0
2	0	0	0	0
3	1	0	0	–1
4	0	0	–1	0
5	0	1	0	0
6	0	0	0	0
7	0	0	1	0
8	0	0	0	1
	Effect of a large-rate of soil application vs no Zn fertiliser	Effect of a double foliar application vs no Zn fertiliser	Effect of adding a large-rate soil application when a single foliar application is made	Effect of adding a double foliar application when a large-rate soil application is made

Table 3. Analysis of Variance tables for crop yield and element concentrations in grain. The four contrasts (C1–C4) represent non-orthogonal components of the fertiliser effect (see Table 2).

		Faisalabad						Islamabad						Pir Sabak					
		df	SS	MS	VR	P	P-adj	df	SS	MS	VR	P	P-adj	df	SS	MS	VR	P	P-adj
Yield	**Replication**	3	460269	153423	2.537	0.069	NA	3	816699	272233	0.67	0.579	NA	3	598758	199586	1.734	0.168	NA
	Variety	1	11586	11586	0.192	0.664	NA	1	1431779	1431779	3.50	0.070	NA	2	903837	451919	3.926	0.024	NA
	Fertiliser	7	329725	47104	0.779	0.608	NA	7	1977788	282541	0.69	0.678	NA	7	795341	113620	0.987	0.448	NA
	C1	1	56220	56220	0.930	0.340	0.819	1	533819	533819	1.31	0.262	0.784	1	8043	8043	0.070	0.792	1.000
	C2	1	2582	2582	0.043	0.837	0.837	1	120409	120409	0.29	0.591	0.915	1	6158	6158	0.054	0.818	1.000
	C3	1	166904	166904	2.760	0.104	0.414	1	231088	231088	0.57	0.458	0.915	1	51888	51888	0.451	0.504	1.000
	C4	1	74453	74453	1.231	0.273	0.819	1	944791	944791	2.31	0.138	0.553	1	1796	1796	0.016	0.901	1.000
	Variety:Fertiliser	7	659629	94233	1.558	0.173	NA	7	324350	46336	0.11	0.997	NA	14	622801	44486	0.386	0.975	NA
	Residuals	45	2721166	60470	NA	NA	NA	32	13073051	408533	NA	NA	NA	69	7942952	115115	NA	NA	NA
Zn	**Replication**	3	935.1	311.7	10.53	0.000	NA	4	387.9	97.0	2.77	0.036	NA	3	250.3	83.4	4.19	0.009	NA
	Variety	1	752.1	752.1	25.41	0.000	NA	1	62.8	62.8	1.79	0.186	NA	2	266.8	133.4	6.70	0.002	NA
	Fertiliser	7	525.3	75.0	2.53	0.028	NA	7	4528.3	646.9	18.46	0.000	NA	7	1738.5	248.4	12.48	0.000	NA
	C1	1	0.2	0.2	0.01	0.939	0.939	1	2.8	2.8	0.08	0.777	1.000	1	10.6	10.6	0.53	0.468	0.663
	C2	1	187.9	187.9	6.35	0.015	0.047	1	1626.8	1626.8	46.42	0.000	0.000	1	642.9	642.9	32.31	0.000	0.000
	C3	1	34.0	34.0	1.15	0.289	0.579	1	0.7	0.7	0.02	0.891	1.000	1	19.0	19.0	0.96	0.332	0.663
	C4	1	204.2	204.2	6.90	0.012	0.047	1	1822.1	1822.1	51.99	0.000	0.000	1	599.9	599.9	30.15	0.000	0.000
	Variety:Fertiliser	7	225.5	32.2	1.09	0.387	NA	7	131.6	18.8	0.54	0.804	NA	14	220.1	15.7	0.79	0.676	NA
	Residuals	45	1332.1	29.6	NA	NA	NA	59	2067.8	35.0	NA	NA	NA	69	1373.1	19.9	NA	NA	NA
Fe	**Replication**	3	91.8	30.6	1.05	0.379	NA	4	160.1	40.0	2.25	0.075	NA	3	740.9	247.0	2.95	0.039	NA
	Variety	1	202.8	202.8	6.97	0.011	NA	1	95.5	95.5	5.36	0.024	NA	2	265.5	132.7	1.59	0.212	NA
	Fertiliser	7	27.2	3.9	0.13	0.995	NA	7	583.9	83.4	4.69	0.000	NA	7	505.6	72.2	0.86	0.540	NA
	C1	1	13.4	13.4	0.46	0.502	1.000	1	13.7	13.7	0.77	0.384	0.769	1	0.2	0.2	0.00	0.958	1.000
	C2	1	1.5	1.5	0.05	0.822	1.000	1	130.2	130.2	7.32	0.009	0.027	1	62.6	62.6	0.75	0.390	1.000
	C3	1	0.0	0.0	0.00	0.997	1.000	1	5.8	5.8	0.32	0.572	0.769	1	32.4	32.4	0.39	0.536	1.000
	C4	1	6.2	6.2	0.21	0.648	1.000	1	359.6	359.6	20.20	0.000	0.000	1	87.9	87.9	1.05	0.309	1.000
	Variety:Fertiliser	7	140.4	20.1	0.69	0.681	NA	7	84.4	12.1	0.68	0.690	NA	14	584.4	41.7	0.50	0.926	NA
	Residuals	45	1309.6	29.1	NA	NA	NA	59	1050.1	17.8	NA	NA	NA	69	5775.6	83.7	NA	NA	NA
Ca	**Replication**	3	2653177	884392	50.58	0.000	NA	4	716364	179091	26.27	0.000	NA	3	618584	206195	55.72	0.000	NA
	Variety	1	728721	728721	41.68	0.000	NA	1	2092	2092	0.31	0.582	NA	2	100799	50400	13.62	0.000	NA
	Fertiliser	7	46059	6580	0.38	0.911	NA	7	78030	11147	1.64	0.143	NA	7	8607	1230	0.33	0.937	NA
	C1	1	6946	6946	0.40	0.532	1.000	1	10564	10564	1.55	0.218	0.436	1	243	243	0.07	0.799	1
	C2	1	9921	9921	0.57	0.455	1.000	1	36961	36961	5.42	0.023	0.093	1	664	664	0.18	0.673	1
	C3	1	681	681	0.04	0.844	1.000	1	3546	3546	0.52	0.474	0.474	1	20	20	0.01	0.942	1
	C4	1	7257	7257	0.42	0.523	1.000	1	18259	18259	2.68	0.107	0.321	1	70	70	0.02	0.891	1
	Variety:Fertiliser	7	78505	11215	0.64	0.719	NA	7	72274	10325	1.51	0.180	NA	14	49170	3512	0.95	0.513	NA
	Residuals	45	786806	17485	NA	NA	NA	59	402214	6817	NA	NA	NA	69	255327	3700	NA	NA	NA
Cd	**Replication**	3	5.0E-04	2.0E-04	3.55	0.022	NA	4	9.0E-04	2.0E-04	4.79	0.002	NA	3	4.0E-04	1.0E-04	6.09	0.001	NA
	Variety	1	2.0E-04	2.0E-04	3.89	0.055	NA	1	0.0E+00	0.0E+00	0.06	0.805	NA	2	6.0E-04	3.0E-04	14.22	0.000	NA
	Fertiliser	7	2.0E-04	0.0E+00	0.71	0.660	NA	7	2.0E-04	0.0E+00	0.65	0.716	NA	7	3.0E-04	0.0E+00	2.31	0.035	NA
	C1	1	0.0E+00	0.0E+00	0.13	0.718	1.000	1	1.0E-04	1.0E-04	1.31	0.258	0.982	1	1.0E-04	1.0E-04	3.59	0.062	0.187
	C2	1	0.0E+00	0.0E+00	0.13	0.716	1.000	1	0.0E+00	0.0E+00	1.03	0.315	0.982	1	2.0E-04	2.0E-04	7.93	0.006	0.025
	C3	1	0.0E+00	0.0E+00	0.09	0.764	1.000	1	1.0E-04	1.0E-04	1.38	0.246	0.982	1	0.0E+00	0.0E+00	1.61	0.208	0.417
	C4	1	0.0E+00	0.0E+00	0.04	0.838	1.000	1	0.0E+00	0.0E+00	0.06	0.815	0.982	1	0.0E+00	0.0E+00	0.88	0.353	0.417
	Variety:Fertiliser	7	2.0E-04	0.0E+00	0.80	0.592	NA	7	5.0E-04	1.0E-04	1.65	0.139	NA	14	2.0E-04	0.0E+00	0.75	0.716	NA
	Residuals	45	2.0E-03	0.0E+00	NA	NA	NA	59	2.8E-03	0.0E+00	NA	NA	NA	69	1.5E-03	0.0E+00	NA	NA	NA

Table 4. Estimated treatment means (± standard error of the mean, SEM) of grain yield and mineral concentration of wheat at three sites under control (Treatment 1, T1) or Zn-fertilised conditions (all units expressed as kg ha^{-1} $ZnSO_4.H_2O$: T2=5 soil; T3=10 soil; T4=0.79 foliar; T5=1.58 foliar; T6=5 soil and 0.79 foliar; T7=10 soil and 0.79 foliar; T8=10 soil and 1.58 foliar).

Site		**Yield (kg/ha)**		**Wheat Grain Concentration (mg/kg)**							
		Mean	**SEM**	**Zn**	**SEM**	**Fe**	**SEM**	**Ca**	**SEM**	**Cd**	**SEM**
Faisalabad	**T1**	2243	87	43.7	1.9	32.1	1.9	410.8	46.8	0.0096	0.0024
	T2	2227	87	43.6	1.9	32.5	1.9	414.2	46.8	0.0076	0.0024
	T3	2361	87	43.9	1.9	34.0	1.9	452.5	46.8	0.0084	0.0024
	T4	2295	87	45.9	1.9	31.8	1.9	435.0	46.8	0.0089	0.0024
	T5	2268	87	50.5	1.9	32.7	1.9	361.0	46.8	0.0109	0.0024
	T6	2231	87	47.6	1.9	32.1	1.9	418.7	46.8	0.0137	0.0024
	T7	2091	87	48.8	1.9	31.8	1.9	448.0	46.8	0.0099	0.0024
	T8	2225	87	51.0	1.9	32.7	1.9	409.9	46.8	0.0077	0.0024
Islamabad	**T1**	3897	261	36.9	1.9	35.8	1.3	742.0	26.1	0.0317	0.0022
	T2	3698	261	37.7	1.9	36.2	1.3	782.0	26.1	0.0272	0.0022
	T3	4199	261	37.6	1.9	34.2	1.3	788.0	26.1	0.0282	0.0022
	T4	3803	261	49.7	1.9	39.7	1.3	802.2	26.1	0.0266	0.0022
	T5	3634	226	54.9	1.9	40.9	1.3	828.0	26.1	0.0286	0.0022
	T6	3735	261	49.5	1.9	40.2	1.3	797.8	26.1	0.0299	0.0022
	T7	4047	242	49.4	2.0	38.2	1.4	842.8	27.5	0.0299	0.0023
	T8	3588	261	56.7	1.9	42.6	1.3	848.4	26.1	0.0275	0.0022
Pir Sabak	**T1**	4626	98	29.8	1.3	40.8	2.6	339.7	17.6	0.0217	0.0013
	T2	4923	98	30.0	1.3	40.5	2.6	322.8	17.6	0.0193	0.0013
	T3	4662	98	31.1	1.3	40.6	2.6	333.3	17.6	0.0181	0.0013
	T4	4728	98	35.6	1.3	39.0	2.6	325.5	17.6	0.0176	0.0013
	T5	4658	98	40.1	1.3	44.0	2.6	329.1	17.6	0.0164	0.0013
	T6	4705	98	38.1	1.3	46.1	2.6	352.9	17.6	0.0173	0.0013
	T7	4635	98	37.3	1.3	41.3	2.6	323.7	17.6	0.0152	0.0013
	T8	4645	98	41.1	1.3	44.4	2.6	336.7	17.6	0.0164	0.0013

Table 5. Mean effect size and standard error (SEM) of each the four contrasts (C1–C4) representing non-orthogonal components of the fertiliser effect (see Table 2).

Site		**Yield (kg/ha)**		**Wheat Grain Concentration (mg/kg)**							
		Mean	**SEM**	**Zn**	**SEM**	**Fe**	**SEM**	**Ca**	**SEM**	**Cd**	**SEM**
Faisalabad	**C1**	118.6	123.0	0.2	2.7	1.8	2.7	41.7	66.1	-0.001	0.003
	C2	25.4	123.0	6.9	2.7	0.6	2.7	-49.8	66.1	0.001	0.003
	C3	-204.3	123.0	2.9	2.7	0.0	2.7	13.1	66.1	0.001	0.003
	C4	-136.4	123.0	7.1	2.7	-1.2	2.7	-42.6	66.1	-0.001	0.003
Islamabad	**C1**	302.1	369.0	0.8	2.6	-1.7	1.9	46.0	36.9	-0.004	0.003
	C2	-262.9	345.2	18.0	2.6	5.1	1.9	86.0	36.9	-0.003	0.003
	C3	244.3	355.6	-0.4	2.7	-1.5	1.9	40.6	37.9	0.003	0.003
	C4	-610.9	369.0	19.1	2.6	8.5	1.9	60.4	36.9	-0.001	0.003
Pir Sabak	**C1**	36.6	138.5	1.3	1.8	-0.2	3.7	-6.4	24.8	-0.004	0.002
	C2	32.0	138.5	10.4	1.8	3.2	3.7	-10.5	24.8	-0.005	0.002
	C3	-93.0	138.5	1.8	1.8	2.3	3.7	-1.8	24.8	-0.002	0.002
	C4	-17.3	138.5	10.0	1.8	3.8	3.7	3.4	24.8	-0.002	0.002