

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

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3 Munir H. Zia<sup>1</sup>, Iftikhar Ahmed<sup>2</sup>, Elizabeth H. Bailey<sup>3</sup>, R. Murray Lark<sup>3</sup>, Scott D. Young<sup>3</sup>,  
4 Nicola M. Lowe<sup>4</sup>, Edward J.M. Joy<sup>5</sup>, Lolita Wilson<sup>3</sup>, Mukhtiar Zaman<sup>6</sup>, Martin R. Broadley<sup>3†</sup>

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6 <sup>1</sup>*Research & Development Department, Fauji Fertilizer Company Ltd, Rawalpindi, Pakistan*

7 <sup>2</sup>*Bioresource Conservation Institute, National Agricultural Research Centre, Islamabad,*  
8 *Pakistan*

9 <sup>3</sup>*School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough,*  
10 *LE12 5RD, UK*

11 <sup>4</sup>*UCLan Research Centre for Global Development, University of Central Lancashire, Preston,*  
12 *PR1 2HE, UK*

13 <sup>5</sup>*Faculty of Epidemiology and Population Health, London School of Hygiene & Tropical*  
14 *Medicine, Keppel St., London, WC1E 7HT, UK*

15 <sup>6</sup>*Rehman Medical College & Abaseen Foundation, 272 Deans Trade Centre, Peshawar Cantt,*  
16 *Peshawar, Pakistan*

17  
18 <sup>†</sup>**Corresponding author:** [martin.broadley@nottingham.ac.uk](mailto:martin.broadley@nottingham.ac.uk)

## 20 **Abstract**

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

27  
28 *Methods:* Grain Zn concentration of *Zincol-2016* and local reference varieties were determined  
29 at three sites of contrasting soil Zn status: Faisalabad (Punjab Province; diethylenetriamine  
30 pentaacetate- (DTPA-)extractable Zn, 1.31 mg kg<sup>-1</sup> soil; gross plot size 13.3 m<sup>2</sup>; n=4; reference  
31 var. *Faisalabad-2008*), Islamabad (Capital Territory; 0.48 mg kg<sup>-1</sup>; 4.6 m<sup>2</sup>; n=5; reference var.  
32 *NARC-2011*), and Pir Sabak (Khyber Pakhtunkhwa, KPK, Province; 0.12 mg kg<sup>-1</sup> soil; 9.1 m<sup>2</sup>;  
33 n=4; reference vars. *Pirsabak-2015*, *Wadhan-2017*). Eight Zn fertiliser treatment levels were  
34 tested using a randomised complete block design: control; soil (5 or 10 kg ha<sup>-1</sup> ZnSO<sub>4</sub>.H<sub>2</sub>O;  
35 33% Zn applied at sowing); foliar (0.79 or 1.58 kg of ZnSO<sub>4</sub>.H<sub>2</sub>O ha<sup>-1</sup> applied as a 250 L ha<sup>-1</sup>  
36 drench at crop booting stage); three soil × foliar combinations.

37  
38 *Results:* At the Faisalabad site, the grain Zn concentration of *Zincol-2016* was greater than  
39 *Faisalabad-2008*, with no yield penalty. *Zincol-2016* did not have larger grain Zn  
40 concentrations than reference varieties used at Islamabad or Pir Sabak sites, which both had a  
41 lower soil Zn status than the Faisalabad site. Foliar Zn fertilisation increased grain Zn  
42 concentration of all varieties at all sites. There were no significant effects of soil Zn fertilisers,  
43 or variety·fertiliser interactions, on grain Zn concentration or yield.

44  
45 *Conclusions:* Environment and management affect the performance of ‘high-Zn’ wheat  
46 varieties, and these factors needs to be evaluated at scale to assess the potential nutritional  
47 impact of Zn biofortified crops. Designing studies to detect realistic effect sizes for new  
48 varieties and crop management strategies is therefore an important consideration. The current  
49 study indicated that nine replicate plots would be needed to achieve 80% power to detect a  
50 25% increase in grain Zn concentration.

## 51 52 53 54 55 **Keywords**

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

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## 61 **Introduction**

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

76

77 Wheat (*Triticum aestivum* L.) is an important cereal crop and a major source of dietary Zn  
78 globally, especially in South Asia where risks of dietary Zn deficiency are likely to be large.  
79 For example, Akhtar (2013) found that the prevalence of Zn deficiency exceeded 40% among  
80 women and children in India and Pakistan, based on surveys of blood plasma/serum Zn status.  
81 In India, Zn concentration in wheat grain, among a panel of 36 diverse genotypes grown in  
82 experimental plots on contrasting soil types, ranged from 24.9–34.8 mg kg<sup>-1</sup> (Khokhar et al.,  
83 2017, 2018). In Pakistan, the concentration of Zn in wheat grain collected from farmers' fields  
84 in 75 locations ranged from 15.1–39.7 mg kg<sup>-1</sup> (Joy et al., 2017). Among a panel of 28 wheat  
85 genotypes of Pakistani origin, grown over two seasons at a single location, grain Zn  
86 concentration ranged from 21.2–33.3 mg kg<sup>-1</sup> with a mean of 27.5 mg kg<sup>-1</sup> (Rehman et al.,  
87 2018b). Assuming a whole-grain Zn concentration of 30 mg kg<sup>-1</sup>, an energy density for wheat  
88 grain of 3400 kcal kg<sup>-1</sup>, and a dietary wheat supply of 517 and 903 kcal capita d<sup>-1</sup> in India and  
89 Pakistan, respectively (FAOSTAT, 2020), the supply of Zn from whole-grain wheat represents  
90 4.6 and 8.0 mg capita<sup>-1</sup> d<sup>-1</sup>, i.e. 45% and 78% of the weighted EARs, for India and Pakistan  
91 respectively.

92

93 The HarvestPlus programme and their partners have used conventional breeding to develop  
94 and release new 'high-Zn' wheat varieties in India and Pakistan, a process known as genetic

95 biofortification (Velu et al., 2015; Singh and Velu, 2017). These new varieties have been  
96 developed from synthetic wheat lines derived from wild wheat relatives, including *Aegilops*  
97 *tauschii* (D genome donor of wheat), *Triticum spelta* and wild *T. dicoccon*, and crosses with *T.*  
98 *durum*. The HarvestPlus target was to enhance the Zn concentration in grain of existing wheat  
99 varieties by 8–12 mg kg<sup>-1</sup>, above a notional baseline whole-grain Zn concentration of 25 mg  
100 kg<sup>-1</sup>, without reducing yield or quality (Velu et al., 2015). In India, ‘high-Zn’ varieties have  
101 been developed and released in the North Eastern Plain Zone (NEPZ): *Abhay* (*Zinc Shakthi*,  
102 *Chitra*), *Akshai* (*BHU-3*) and *BHU-6*, and in the North Western Plain Zone (NWPZ): *WB02*  
103 and *HPBW-01* (Velu et al., 2015; Singh and Velu, 2017). In Pakistan, a ‘high-Zn’ wheat variety  
104 *Zincol-2016*, developed by National Agriculture Research System (NARS) from a background  
105 *NARC-2011* variety, was released by the Pakistan Agriculture Research Council (PARC) in  
106 2016.

107

108 In addition to genetic approaches, grain Zn concentration in wheat can also be increased with  
109 Zn-containing fertilisers, a process termed agronomic biofortification or agro-fortification  
110 (Cakmak, 2008; White and Broadley, 2009; Zhao et al., 2019). In a review of nine published  
111 field studies, Joy et al. (2015b) noted that foliar Zn (ZnSO<sub>4</sub>) fertilisers, applied as a drench to  
112 field-grown wheat, increased the whole-grain Zn concentration by a median of 63%. Soil-  
113 applied Zn fertilisers can also increase grain Zn concentrations, albeit to a much lesser extent  
114 than foliar-applied Zn fertilisers but may also increase crop yield in some settings (Cakmak  
115 2008; Zou et al., 2012). In a review of 14 published field studies, soil-applied Zn fertilisers  
116 increased whole-grain Zn concentration of field-grown wheat by a median of 19% (Joy et al.,  
117 2015b). In Pakistan, soil-applied Zn fertilisers led to an increase in the Zn concentration of  
118 whole-grain *chapati* flatbread, from 18±2 to 24±2 mg kg<sup>-1</sup> (mean±SD) (Ahsin et al., 2019). In  
119 India, wheat agro-fortified with foliar Zn fertiliser and supplied as a Zn-enriched flour for six  
120 months to women and children aged from 4 to 6 years resulted in a 17% and 40% reduction in  
121 self-reported incidences of pneumonia and vomiting, respectively (Sazawal et al., 2018).

122

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

128 diethylenetriamine pentaacetate- (DTPA-) extractable Zn, Hussain et al. (2018) reported that  
129 *Zincol-2016* (~22 mg kg<sup>-1</sup>) had a larger grain Zn concentration than *Faisalabad-2008* (~18 mg  
130 kg<sup>-1</sup>). When Zn fertiliser was added to soils, the differences in grain Zn between *Zincol-2016*  
131 (~36 mg kg<sup>-1</sup>) and *Faisalabad-2008* (~25 mg kg<sup>-1</sup>) increased markedly. In a pot study by  
132 Yousaf et al. (2019), *Zincol-2016* (33.9 mg kg<sup>-1</sup>) had a much larger grain Zn concentration than  
133 *Faisalabad-2008* (23.8 mg kg<sup>-1</sup>) in unfertilised soils. However, genotypic differences were not  
134 evident when foliar or soil Zn fertilisers were added and which increased the grain Zn  
135 concentration in both varieties. In a pot study by Yaseen and Hussain (2020), *Zincol-2016* had  
136 a greater grain Zn concentration than a reference variety, *Jauhar-2016*, when Zn fertilisers  
137 were added to alkaline calcareous soils although there was no genotypic difference in grain Zn  
138 concentration under control conditions. The aim of this study was to quantify the potential  
139 contribution of *Zincol-2016* to improving the dietary supply of Zn under experimental field  
140 conditions. Field experiments were established in Pakistan at three sites of contrasting soil Zn  
141 status, where *Zincol-2016* was grown in replicated plots and compared with local reference  
142 lines, with and without soil and/or foliar Zn fertilisers.

143

144

## 145 **Materials and Methods**

### 146 *Site selection and characterisation*

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

153

### 154 *Experimental design and layout*

155 The experiments sought to test the effect of variety and Zn fertilisers on wheat grain yields and  
156 Zn concentration at each of the three sites. The choice of variety was site-specific, so that the  
157 performance of *Zincol-2016* could be compared directly with reference varieties used routinely  
158 by farmers in the same locations (Table 1). At all sites, eight Zn fertiliser treatment levels were  
159 tested (Table 2): control; soil-applied (5 or 10 kg ha<sup>-1</sup> ZnSO<sub>4</sub>.H<sub>2</sub>O; 33% Zn applied at sowing);  
160 foliar-applied (0.79 or 1.58 kg of ZnSO<sub>4</sub>.H<sub>2</sub>O ha<sup>-1</sup> applied as a 250 L ha<sup>-1</sup> drench at crop booting  
161 stage, Zadoks' scale 45-50; Zadoks et al., 1974); and three combinations of soil- and foliar-

162 applied ZnSO<sub>4</sub>.H<sub>2</sub>O comprising 5+0.79 kg ha<sup>-1</sup> soil+foliar, 10+0.79 kg ha<sup>-1</sup> soil+foliar, and  
163 10+1.58 kg ha<sup>-1</sup> soil+foliar. A complete randomised block design was adopted at each site,  
164 comprising four replicates at Faisalabad and Pir Sabak, and five replicates at Islamabad. Layout  
165 details are provided in Supplementary Information.

166

### 167 *Agronomy*

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

175

176 General fertiliser applications comprised basal phosphorus (di-ammonium phosphate, P<sub>2</sub>O<sub>5</sub>  
177 46%) at 115 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup>, and potassium (muriate of potash, K<sub>2</sub>O 60%) at 75 kg K<sub>2</sub>O ha<sup>-1</sup> at  
178 Faisalabad and Pir Sabak. Potassium was not applied at Islamabad as soil testing indicated  
179 adequate potassium status. Basal fertilisers were applied at time of soil preparation, prior to  
180 sowing. Nitrogen (urea at 110 kg ha<sup>-1</sup>) was split in to two halves, one half-applied at time of  
181 first irrigation (Zadoks' scale ~25) whereas the remaining half at Zadoks' scale ~40). Soil-  
182 applied Zn fertiliser was broadcast uniformly in the designated treatment plot(s) and  
183 incorporated into the soil before sowing. The foliar treatment for Zn fertilisers was applied in  
184 the early morning hours to reduce risk of leaf-scorch.

185

### 186 *Measurements of yield and yield components*

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

194

### 195 *Determining grain concentration of Zn and other elements*

196 Grain digestion and elemental analysis methods are described in Khokhar et al. (2018, 2020).  
197 Briefly, approximately 10 grains (whole-grain) were dried, weighed, and soaked in 3 mL 70%  
198 Trace Analysis Grade (TAG) HNO<sub>3</sub> and 2 mL H<sub>2</sub>O<sub>2</sub>, at room temperature overnight, in  
199 perfluoroalkoxy (PFA) tubes (Anton Paar GmbH, Graz, Austria). The tubes were then placed  
200 into polyethylethylketone (PEEK) pressure jackets and digested in a Multiwave 3000  
201 microwave system with a 48-vessel MF50 rotor (Anton Paar GmbH). Whole-grain Zn  
202 concentration was determined by inductively coupled plasma-mass spectrometry (ICP-MS;  
203 Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany). The Zn  
204 recovery from nine samples of a Certified Reference Material (CRM; Wheat flour SRM 1567b,  
205 NIST, Gaithersburg, MD, US; 11.61 mg kg<sup>-1</sup>) was 94.4% (first run) and 91.2% (second run).  
206 The Limit of Detection (LOD) for Zn, equivalent to 3 times the standard deviation (SD) of the  
207 concentrations of all of the operational blanks and a notional dry weight of 0.35 g was 4.45 and  
208 2.47 mg kg<sup>-1</sup> for the first and second analysis runs, respectively. The full range of elements  
209 reported from the ICP-MS were Ag, Al, As, B, Ba, Be, Ca, Cd, Cr, Co, Cs, Cu, Fe K, Li, Mg,  
210 Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, Tl, U, V, and Zn (Supplementary Information). Data  
211 for Zn, Fe, Cd and Ca are reported here.

212

### 213 *Data analyses*

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

222

223 If the main effect of fertiliser appeared significant, then it was examined further by testing a  
224 set of contrasts among levels of the fertiliser factor against the Residual Mean Square (RMS)  
225 for the overall ANOVA. The treatments used in the study do not naturally partition into a set  
226 of informative orthogonal contrasts. Therefore, we examined a set of non-orthogonal contrasts,  
227 controlling the family-wise error rate with Holm's modification of Bonferroni's method (Holm,  
228 1979), and we reported adjusted p-values. Sokal and Rohlf (2012) recommend this approach  
229 when examining non-orthogonal contrasts. Given that power is lost for each additional test,

230 four informative contrasts were selected (Table 2) and the treatment by variety interaction was  
231 not partitioned for the contrast analyses. Effect sizes for all four contrasts, and a (pooled)  
232 standard error are reported. R scripts are provided in Supplementary Information).

233  
234 The contrasts were defined before any data from the experiment were examined. The rationale  
235 for this choice of contrasts was to explore the largest respective effects of soil application and  
236 foliar application (C1 and C2) relative to the no-fertiliser control, and then to examine the  
237 evidence for an incremental improvement from a large-rate soil application when a single foliar  
238 application is in use (C3, “with a standard foliar application, is there any benefit in applying  
239 Zn to the soil as well?”), and from adding a double foliar application when a large-rate soil  
240 application is in use (C4, “when applying Zn to the soil, is there a supplementary benefit of  
241 applying a foliar dose as well?”). As is noted above, these 4 contrasts, each with 1 degree of  
242 freedom, are not orthogonal. That is to say the contrasts are not independent of each other, and  
243 so do not give independent tests on components of the sum of squares for treatments.

244

245

## 246 **Results**

247 The outputs of the ANOVA for treatment factors, their interactions, and selected contrasts, for  
248 the variates of yield and grain Zn, Fe, Ca and Cd concentration are presented in Table 3.  
249 Arithmetic means across the plots for these same variates are plotted in Figure 1; individual  
250 plot-level data, including yield components, are provided as Supplementary Information.  
251 Fertiliser treatment means, and the effects sizes of the chosen contrasts, are presented in Tables  
252 4 and 5, respectively. The interpretation of the effects sizes is conditional on the signs (i.e. a  
253 positive value for C1 would indicate that the mean for the soil Zn treatment is larger than the  
254 mean for the control). The standard error is obtained from the pooled RMS, so it is the same  
255 where replication sizes are equal.

256

### 257 *Grain yield*

258 At all three sites, there was no evidence to reject the null hypothesis of no effect of Zn fertiliser  
259 application, or variety·Zn fertiliser interaction, on yield (Table 3). The lack of yield responses  
260 to Zn fertilisers was unexpected given that wheat is generally responsive to Zn fertilisers on  
261 calcareous soil types in Pakistan (e.g. Joy et al., 2017; Rehman et al., 2018a; Asif et al., 2019).  
262 At the Faisalabad and Islamabad sites, there was no evidence to reject the null hypothesis of  
263 no difference in mean yield among varieties, however, there was some evidence to reject this



264 null hypothesis at the Pir Sabak site ( $p=0.024$ ; Table 3), with *Wadhan-2017* having a slightly  
265 greater yield than *Pirsabak-2015* and *Zincol-2016*. The overall grain yield of *Zincol-2016* and  
266 *Faisalabad-2008* was ~50% of those observed for *Zincol-2016* and reference varieties at  
267 Islamabad and Pir Sabak. The soil texture at the Faisalabad site is “sandy loam” where one  
268 would always expect a yield penalty compared to the “silt loam” textured soils at the other  
269 locations. There was also a yellow rust attack at the time of grain formation/development at  
270 the Faisalabad site and surrounding area in 2019.

271

#### 272 *Grain zinc concentration*

273 There was strong evidence to reject the null hypothesis of no difference in grain Zn  
274 concentration between the varieties at the Faisalabad ( $p<0.001$ ) and Pir Sabak ( $p=0.002$ ) sites,  
275 (Table 3). At Faisalabad, *Zincol-2016* had a consistently larger grain Zn concentration than  
276 *Faisalabad-2008*; a difference of ~16% averaged across all 8 fertiliser treatment levels (Figure  
277 1; Supplementary Information). At Pir Sabak, grain Zn concentration decreased in the order  
278 *Wadhan-2017* > *Zincol-2016* > *Pirsabak-2015*. At Islamabad, there was no evidence to reject  
279 the null hypothesis of no difference in grain Zn concentration between the varieties ( $p=0.186$ ;  
280 Table 3).

281

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

293

#### 294 *Grain iron concentration*

295 There was evidence to reject the null hypothesis of no difference among the varieties with  
296 respect to grain Fe concentration at the Faisalabad ( $p=0.011$ ) and Islamabad ( $p=0.024$ ) sites.  
297 At Faisalabad, *Zincol-2016* had a larger grain Fe concentration than *Faisalabad-2008*; a

298 difference of ~12% averaged across all 8 fertiliser treatment levels (Figure 1; Supplementary  
299 Information). At Islamabad, *Zincol-2016* had a larger grain Fe concentration than *NARC-2011*;  
300 a difference of ~6% averaged across all 8 fertiliser treatment levels (Figure 1; Supplementary  
301 Information). At the Pir Sabak site, the null hypothesis of no difference among the varieties  
302 with respect to grain Fe concentration was retained ( $p=0.212$ ; Table 3).

303

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

312

#### 313 *Grain calcium concentration*

314 There was evidence to reject the null hypothesis of no difference among the varieties with  
315 respect to grain Ca concentration at the Faisalabad ( $p<0.001$ ) and Pir Sabak ( $p<0.001$ ) sites,  
316 (Table 3). At Faisalabad, *Faisalabad-2008* had a larger grain Ca concentration than *Zincol-*  
317 *2016*; a difference of ~68% averaged across all 8 fertiliser treatment levels (Figure 1;  
318 Supplementary Information). At Pir Sabak, *Zincol-2016* had a larger grain Ca concentration  
319 than *Wadhan-2017*; a difference of ~20% averaged across all 8 fertiliser treatment levels  
320 (Figure 1; Supplementary Information). However, at Pir Sabak, *Pirsabak-2015* had a larger  
321 grain Ca concentration than *Zincol-2016*; a difference of ~5% averaged across all 8 fertiliser  
322 treatment levels. At the Islamabad site, there was no evidence for varietal differences in grain  
323 Ca concentration ( $p=0.582$ ; Table 3). There was no evidence of any effects of Zn fertiliser, or  
324 variety·Zn fertiliser interactions, on grain Ca concentration at any of the three sites (Table 3).

325

#### 326 *Grain cadmium concentration*

327 There was evidence to reject the null hypothesis of no difference among the varieties with  
328 respect to grain Cd concentration at the Pir Sabak site ( $p<0.001$ ), (Table 3). *Zincol-2016* had a  
329 larger grain Cd concentration than *Pirsabak-2015*; a difference of ~34% averaged across all  
330 fertiliser treatments. However, *Wadhan-2017* had a larger grain Cd concentration than *Zincol-*  
331 *2016*; also a difference of ~34% averaged across all 8 fertiliser treatment levels (Figure 1;

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

339

340

### 341 **Discussion**

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

354

355 The experiments reported in this current study were not designed to test for effects of site on  
356 varietal performance. However, it is noteworthy that soils at Faisalabad had a larger  
357 concentration of DTPA-extractable soil Zn than the soils at the other two sites. Several studies  
358 have reported significant positive correlations between DTPA-extractable soil Zn  
359 concentration and wheat grain Zn concentrations under field conditions. For example, in a  
360 recent study in China, wheat grain Zn concentration correlated positively with soil available  
361 Zn in single wheat, wheat-maize, and rice-wheat cropping systems (Huang et al., 2019). Similar  
362 positive correlations have also been reported under field conditions in Iran (Karami et al.,  
363 2009), France (Oury et al., 2006), and Slovakia (Krauss et al., 2002). However, whilst available  
364 soil Zn clearly has predictive power, wheat grain Zn concentration is a complex trait which is

365 influenced by many additional soil, varietal, and climatic factors (Karami et al., 2009; Huang  
366 et al., 2019).

367

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

377

378 There were no significant effects of soil Zn fertilisers, or variety·fertiliser interactions, on grain  
379 Zn concentration at any of the sites. The use of soil Zn fertilisers has been reported to increase  
380 wheat grain Zn concentration in other field studies, albeit to a smaller extent than foliar Zn  
381 fertilisers (Joy et al., 2015b). For example, an average increase in grain Zn concentration of  
382 12% was reported across 23 site-year combinations, spanning seven countries (Zou et al.,  
383 2012). Soil Zn fertilisers have also been reported to increase available Zn, for example, in a  
384 field study in Punjab Province, Pakistan, Ahsin et al. (2019) reported greater soil concentrations  
385 of DTPA-extractable Zn ( $1.1 \pm 0.1 \text{ mg kg}^{-1}$ ; mean  $\pm$  standard deviation, SD) in soils treated with  
386 Zn, than when no Zn fertilisers were applied ( $0.8 \pm 0.1 \text{ mg kg}^{-1}$ ). Soil applications of Zn  
387 fertilisers have specifically been shown to be effective at increasing the grain Zn concentration  
388 of *Zincol-2016* in pot experiments (Yousaf et al., 2019; Yaseen and Hussain, 2020). However,  
389 further research is needed to understand the potential value of longer-term soil fertility building  
390 with soil Zn fertilisers with new Zn-biofortified wheat varieties under field conditions,  
391 including the potential for multi-year effects, and the use of other nutrients to augment Zn  
392 uptake and translocation to grain. For example, farmer management such as an increased use  
393 of nitrogen fertilisers (Xue et al., 2012) and organic inputs (Wood et al., 2018) can increase  
394 wheat grain Zn concentration in field settings. Similarly, an increased use of organic materials  
395 (Manzeke et al, 2019) and nitrogen fertilisers (Manzeke et al., 2014; 2020) has been reported  
396 to increase grain Zn concentration in field-grown maize in smallholder farming systems.

397

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

408

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

417

418 In terms of dietary Zn intake, even small changes in Zn concentration in staple foods can  
419 translate into large effects on estimates of population-level prevalence of Zn deficiency. In the  
420 current study, an increase in grain Zn concentration of 1 mg kg<sup>-1</sup> would increase dietary Zn  
421 intake by 0.27 mg capita<sup>-1</sup> d<sup>-1</sup>, assuming a current dietary intake of Zn from wheat of 8 mg  
422 capita<sup>-1</sup> d<sup>-1</sup> arising from a grain consumption of 266 g capita<sup>-1</sup> d<sup>-1</sup> in Pakistan. An increase in  
423 grain Zn concentration of 4 mg kg<sup>-1</sup> would increase dietary intakes by an average of >1 mg  
424 capita<sup>-1</sup> d<sup>-1</sup> which is >10% of the EAR for Zn of ~10.3 mg capita<sup>-1</sup> d<sup>-1</sup> in Pakistan (Kumssa et  
425 al., 2015). There is therefore clear scope for the agriculture sector to mitigate a projected 9%  
426 decrease in wheat grain Zn concentration arising due to greater atmospheric CO<sub>2</sub> (mid-21<sup>st</sup>  
427 Century scenario of 550 ppm; Smith and Myers, 2018). Intriguingly, a ~30% larger maize grain  
428 Zn concentration attributed to a particular Vertisol soil type in Malawi (Chilimba et al., 2011;  
429 Joy et al., 2015a), corresponded with a larger inherent dietary Zn intake of 1.6 mg capita<sup>-1</sup> d<sup>-1</sup>  
430 based on composite dietary analyses among smallholder farming communities (Siyame et al.,

431 2013). However, it was not possible to link this elevated Zn intake among farmers growing  
432 crops on the Vertisols to differences in Zn status based on biomarkers, likely because Zn  
433 concentrations in blood plasma/serum are under tight homeostatic control. Similarly, Sazawal  
434 et al. (2018) did not observe a change in biomarkers of Zn status among individuals consuming  
435 wheat grain with a 50% greater Zn concentration, following foliar Zn fertiliser application,  
436 although self-reported health improvements were noted over their six-month study period.  
437 These studies highlight the need to consider dietary Zn intake as part of decision support for  
438 managing Zn deficiency.

439

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

453

454 Beyond Zn, wheat is an important dietary source of a range of other mineral micronutrients.  
455 Positive correlations between grain Zn and Fe concentrations have been reported when  
456 different varieties of wheat are being phenotyped (e.g. Khokhar et al., 2020). Interventions to  
457 increase dietary Zn intake through breeding might therefore have added nutritional benefits.  
458 For Fe, *Zincol-2016* had a larger grain Fe concentration than the local varieties at two of the  
459 three sites, Faisalabad (*cf. Faisalabad-2008*) and Islamabad (*cf. NARC-2011*), but not at Pir  
460 Sabak. For Ca, another important human micronutrient, *Zincol-2016* had a larger grain Ca  
461 concentration than *Faisalabad-2008* and *Wadhan-2017*, at Faisalabad and Pir Sabak,  
462 respectively. In contrast, *Zincol-2016* had a smaller grain Ca concentration than *Pirsabak-2015*  
463 at the Pir Sabak site. Whilst there was limited evidence that Zn fertiliser applications affected

464 grain Fe (or Ca) concentrations, the site-specific varietal responses reported in this study show  
465 the importance of phenotyping grain for multiple nutrient elements during biofortification  
466 breeding programmes.

467

468 The grain concentrations of 19 mineral elements are reported in this current study  
469 (Supplementary Information). Beyond the traits of grain Zn, Fe, and Ca concentration, which  
470 are heritable and amenable to crop breeding (Khokhar et al., 2018), the grain concentration of  
471 other essential dietary micronutrients, such as selenium (Se), have low heritability and are  
472 influenced to a far greater extent by the soil environment in which the crop is grown (White  
473 and Broadley, 2009). Interestingly, grain Se concentration across all plots at Faisalabad  
474 (median 0.082 mg kg<sup>-1</sup>; range 0.060–0.119) was almost five-fold greater than at Pir Sabak  
475 (median 0.017 mg kg<sup>-1</sup>; range 0.008–0.033), dwarfing any potential effect of variety or  
476 agronomy in the current study. It will be interesting to discover if further evidence emerges of  
477 systematic – and nutritionally important – spatial variation in grain Se concentration across the  
478 major wheat growing areas of Pakistan, as has been observed in sub-Saharan Africa for wheat  
479 and *teff* (*Eragrostis tef* (Zucc.) Trotter; Gashu et al., 2020), and also for maize (Ligowe et al.,  
480 2020).

481

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

493

494 In addition to potentially toxic elements, it will also be important to determine how G × E × M  
495 factors will influence the concentration of phytate and other anti-nutritional factors which can  
496 inhibit the bioavailability of Zn, Fe, and other mineral nutrients in the human gut. Anti-

497 nutritional factors were not considered in the current study. Interestingly, in the recent study of  
498 Yaseen and Hussain (2020), using alkaline calcareous soils, there were no genotypic  
499 differences in grain Zn or phytate concentration under control conditions between *Zincol-2016*  
500 and the reference variety *Jauhar-2016*. However, *Zincol-2016* had a greater grain Zn  
501 concentration and a lower phytate concentration than *Jauhar-2016* when Zn fertilisers were  
502 added, indicating that the bioavailable Zn would be greater in *Zincol-2016*.

503

504

## 505 **Conclusions**

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

516

517

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529

## 530 **Author contribution statement**



531 MHZ, RML, and MRB designed the study. MHZ, IA, EHB, SDY, and LW conducted the field  
532 experiments and sample analyses. MHZ, RML, and MRB analysed the data. MHZ, RML,  
533 EJMJ, and MRB wrote the manuscript. All authors edited and approved the final version of the  
534 manuscript. MHZ, NML (BiZiFED Principal Investigator), MZ, and MRB secured funding for  
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537

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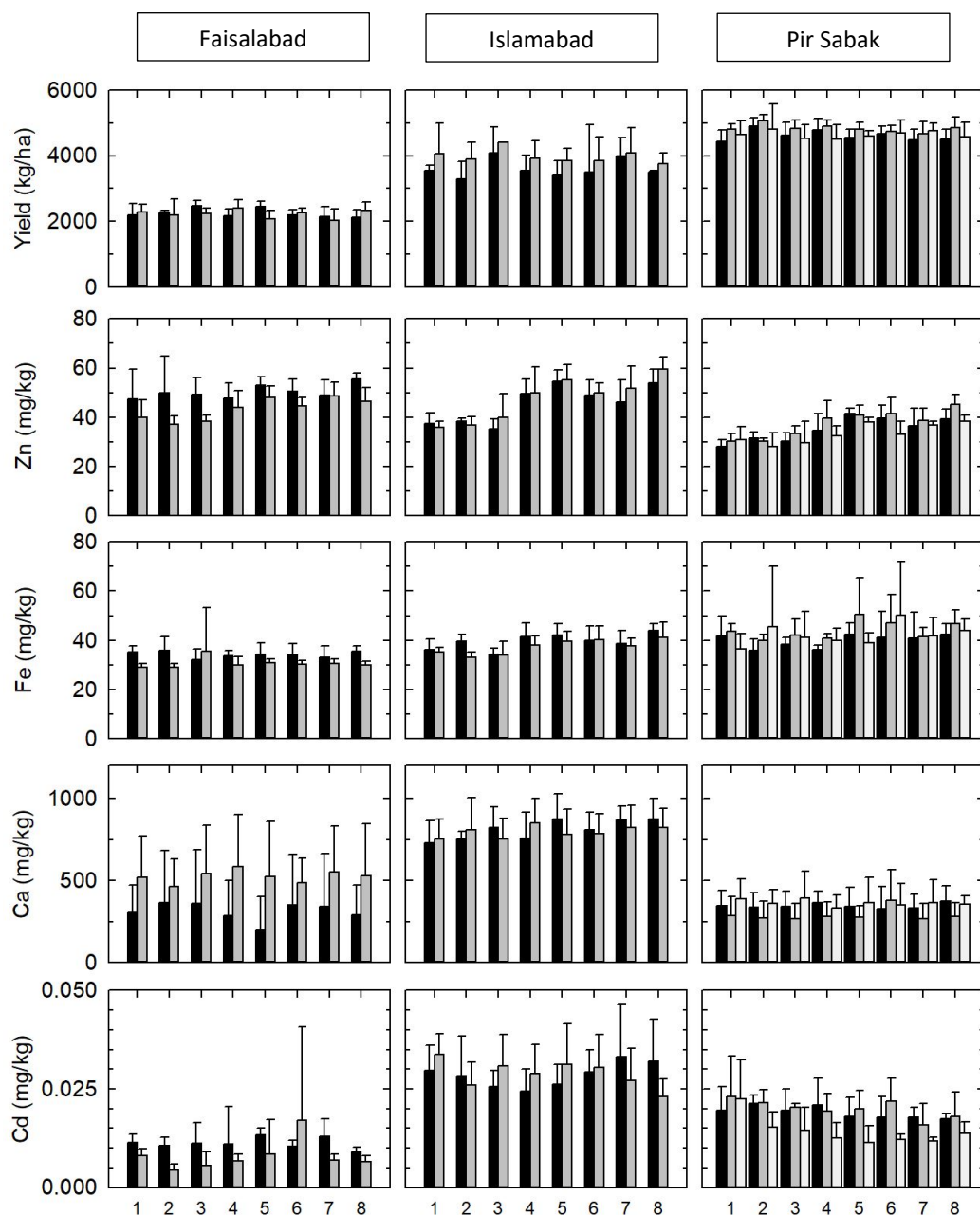
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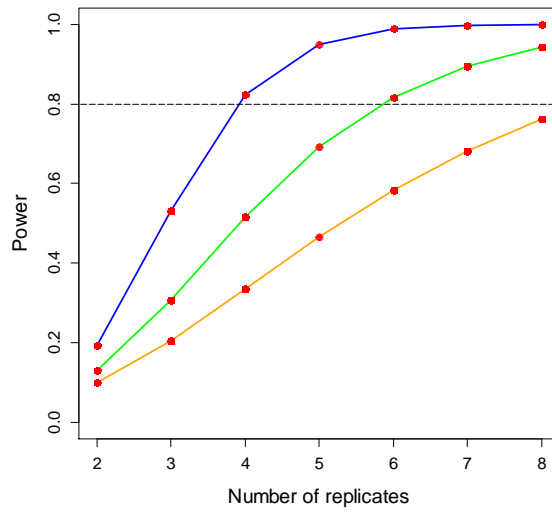
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703 **Figure 1.** Arithmetic means ( $\pm$  standard deviation, SD) of grain yield and mineral  
 704 concentration of wheat at three sites under control (Treatment 1, T1) or Zn-fertilised conditions  
 705 (all units expressed as  $\text{kg ha}^{-1}$   $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ : T2=5 soil; T3=10 soil; T4=0.79 foliar; T5=1.58  
 706 foliar; T6=5 soil and 0.79 foliar; T7=10 soil and 0.79 foliar; T8=10 soil and 1.58 foliar). Black  
 707 bars are *Zincol-2016*; grey bars are local reference varieties (*Faisalabad-2008* at Faisalabad;  
 708 *NARC-2011* at Islamabad; *Wadhan-2017* and *Pirsabak-2015* – lighter grey – at Pir Sabak).

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

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720

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

723

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

724 <sup>1</sup>Soil pH<sub>1:2.5</sub> (soil:water, NF X31-103 1988; AFNOR, 1994)

725 <sup>2</sup>Walkley (1947)

726 <sup>3</sup>Lindsay and Norvell (1978)

727

728 **Table 2.** Contrasts tested in this study four contrasts (C1–C4) represent non-orthogonal  
 729 components of the fertiliser effect. Treatment 1 (T1) represents control conditions with no Zn  
 730 fertilisers; T2-8 represent Zn-fertilised conditions (all units expressed as kg ha<sup>-1</sup> ZnSO<sub>4</sub>.H<sub>2</sub>O:  
 731 T2=5 soil; T3=10 soil; T4=0.79 foliar; T5=1.58 foliar; T6=5 soil and 0.79 foliar; T7=10 soil  
 732 and 0.79 foliar; T8=10 soil and 1.58 foliar).

733

	<b>Contrast</b>			
<b>Treatment</b>	<b>C1</b>	<b>C2</b>	<b>C3</b>	<b>C4</b>
<b>1</b>	-1	-1	0	0
<b>2</b>	0	0	0	0
<b>3</b>	1	0	0	-1
<b>4</b>	0	0	-1	0
<b>5</b>	0	1	0	0
<b>6</b>	0	0	0	0
<b>7</b>	0	0	1	0
<b>8</b>	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

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735

736 **Table 3.** Analysis of Variance tables for crop yield and element concentrations in grain. The  
 737 four contrasts (C1–C4) represent non-orthogonal components of the fertiliser effect (see Table  
 738 2).  
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		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
<b>Yield</b>	<b>Replication</b>	3	460269	153423	2.537	0.069	NA	3	816699	272233	0.67	0.579	NA	3	598758	199586	1.734	0.168	NA
	<b>Variety</b>	1	11586	11586	0.192	0.664	NA	1	1431779	1431779	3.50	0.070	NA	2	903837	451919	3.926	0.024	NA
	<b>Fertiliser</b>	7	329725	47104	0.779	0.608	NA	7	1977788	282541	0.69	0.678	NA	7	795341	113620	0.987	0.448	NA
	<b>C1</b>	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
	<b>C2</b>	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
	<b>C3</b>	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
	<b>C4</b>	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
	<b>Variety:Fertiliser</b>	7	659629	94233	1.558	0.173	NA	7	324350	46336	0.11	0.997	NA	14	622801	44486	0.386	0.975	NA
	<b>Residuals</b>	45	2721166	60470	NA	NA	NA	32	13073051	408533	NA	NA	NA	69	7942952	115115	NA	NA	NA
<b>Zn</b>	<b>Replication</b>	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
	<b>Variety</b>	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
	<b>Fertiliser</b>	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
	<b>C1</b>	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
	<b>C2</b>	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
	<b>C3</b>	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
	<b>C4</b>	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
	<b>Variety:Fertiliser</b>	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
	<b>Residuals</b>	45	1332.1	29.6	NA	NA	NA	59	2067.8	35.0	NA	NA	NA	69	1373.1	19.9	NA	NA	NA
<b>Fe</b>	<b>Replication</b>	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
	<b>Variety</b>	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
	<b>Fertiliser</b>	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
	<b>C1</b>	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
	<b>C2</b>	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
	<b>C3</b>	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
	<b>C4</b>	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
	<b>Variety:Fertiliser</b>	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
	<b>Residuals</b>	45	1309.6	29.1	NA	NA	NA	59	1050.1	17.8	NA	NA	NA	69	5775.6	83.7	NA	NA	NA
<b>Ca</b>	<b>Replication</b>	3	2653177	884392	50.58	0.000	NA	4	716364	179091	26.27	0.000	NA	3	618584	206195	55.72	0.000	NA
	<b>Variety</b>	1	728721	728721	41.68	0.000	NA	1	2092	2092	0.31	0.582	NA	2	100799	50400	13.62	0.000	NA
	<b>Fertiliser</b>	7	46059	6580	0.38	0.911	NA	7	78030	11147	1.64	0.143	NA	7	8607	1230	0.33	0.937	NA
	<b>C1</b>	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
	<b>C2</b>	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
	<b>C3</b>	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
	<b>C4</b>	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
	<b>Variety:Fertiliser</b>	7	78505	11215	0.64	0.719	NA	7	72274	10325	1.51	0.180	NA	14	49170	3512	0.95	0.513	NA
	<b>Residuals</b>	45	786806	17485	NA	NA	NA	59	402214	6817	NA	NA	NA	69	255327	3700	NA	NA	NA
<b>Cd</b>	<b>Replication</b>	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
	<b>Variety</b>	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
	<b>Fertiliser</b>	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
	<b>C1</b>	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
	<b>C2</b>	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
	<b>C3</b>	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
	<b>C4</b>	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
	<b>Variety:Fertiliser</b>	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
	<b>Residuals</b>	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

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745 **Table 4.** Estimated treatment means ( $\pm$  standard error of the mean, SEM) of grain yield and  
746 mineral concentration of wheat at three sites under control (Treatment 1, T1) or Zn-fertilised  
747 conditions (all units expressed as kg ha<sup>-1</sup> ZnSO<sub>4</sub>.H<sub>2</sub>O: T2=5 soil; T3=10 soil; T4=0.79 foliar;  
748 T5=1.58 foliar; T6=5 soil and 0.79 foliar; T7=10 soil and 0.79 foliar; T8=10 soil and 1.58  
749 foliar).

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

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

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

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