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Combined Zinc and Nitrogen Fertilization in Different Bread Wheat Genotypes Grown under Mediterranean Conditions

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The combined application of nitrogen (N) and zinc (Zn) appears to be a promising agronomic strategy for the biofortification with Zn. To evaluate such efficiency, a field experiment was conducted in south-eastern Portugal under Zn-deficient soil. Four advanced breeding lines and two commercial varieties of bread wheat (*Triticum aestivum* L.) were fertilized with five treatments: i) control, ii) two foliar Zn applications, iii) one foliar Zn+N application, iv) soil and two foliar Zn applications, and v) soil and one foliar Zn+N application. Grain Zn content varied greatly across treatments and INIAV-1 and the commercial varieties were the most interesting cultivars in all the treatments. Grain Zn concentrations higher than the target level of 38 mg Zn kg^{-1} were obtained only when two foliar Zn applications were applied, alone or in combination with soil Zn applications, and grain Zn bioavailability also was more adequate (phytate:Zn ratios similar to 15). Soil Zn application resulted in grain yield increases between 7–10%, which virtually offset the extra application cost. The combined soil and two foliar treatment could be a good option for biofortifying bread wheat under Zn-deficient soils.

Keywords: zinc deficiency, urea, agronomic biofortification, genetic biofortification, zinc fertilizers

Introduction

Zinc (Zn) is an essential micronutrient in plants, animals and humans. However, more than 30% of the world's population is Zn deficient, including around 10% of Spain and Portugal (Hotz and Brown 2004; WHO 2009). A deficient Zn intake by humans is associated with severe health complications, including impairments of physical growth, immune system and learning ability, combined with increased risk of infections, DNA damage and cancer development (Hotz and Brown 2004; Levenson and Morris 2011). The European Recommended Dietary Intake (RDI) of Zn for humans is 15 mg Zn day^{-1} (Elmadfa 2009). However, as Gomez-Coronado et al. (2016) reviewed, the intake of about 56% of the Spanish population is below of two thirds of the RDA (Sanchez et al.

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2009), mainly in the institutionalised elderly (Mensink et al. 2013). So, according to such values, daily Zn intake, especially in the older population, should be increased to reach the recommended values.

Food consumption provides the principal route of Zn intake in most of the population, being Zn contents directly related to the level of bioavailable Zn in the soil from which the food was derived (Cakmak et al. 2010), being 0.5 mg kg^{-1} the limiting concentration of wheat growth and yield as well as grain Zn contents (Sims and Johnson 1991). Agronomic Zn biofortification has been shown to be effective in increasing Zn in common dietary foodstuff (Zou et al. 2012; Ghasemi et al. 2013; Wang et al. 2015; Gomez-Coronado et al. 2016). Cereals, being consumed in large amounts, could constitute a major source of Zn in the diet, estimated by Terrés et al. (2001) in about 25%. Bread wheat (*Triticum aestivum* L.) is the most consumed cereal in the European countries. The total grain Zn concentration found in south-western Portugal ranged between 20 and 30 mg Zn kg^{-1} (Galinha et al. 2013). These levels are lower than the target level, established by the HarvestPlus program (www.harvestplus.org) in 38 mg Zn kg^{-1} or by Wang et al. (2012) in 40 mg Zn kg^{-1}, to achieve a sufficient Zn status in humans. The grain Zn content is not the only important parameter, the knowledge of their bioavailability is also crucial. Phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate) is one of the major drawbacks limiting the nutritional quality of cereals. It binds with Zn to form insoluble complexes that hinder Zn absorption in the human intestine. To determine Zn bioavailability in food, molar ratio of phytate: Zn is one of the most accepted methods, being ratios greater than 15 associated with Zn deficiency (Gargari et al. 2007). Most cereals and their products contain high ratios, e.g. between 25 and 34, being not able to be totally absorbed by humans (Welch and Graham 2002).

To increase the daily Zn intake and approximate it to the RDI levels and improve their bioavailability, in soils with low chemical availability in Zn, the combination of genetic and agronomic biofortification is considered to be the most long-term sustainable and cost-efficient strategy (Alloway 2009; Joy et al. 2015; Gomez-Coronado et al. 2016). Foliar Zn application, alone or in combination with soil Zn application, is the most efficient technique to increase significantly wheat grain Zn content, in both Zn-sufficient and Zn-deficient soils (Cakmak et al. 2010; Zhang et al. 2012; Zou et al. 2012). In fact, previous studies have shown that foliar Zn application is effective decreasing the phytate:Zn ratio and increasing estimated Zn bioavailability (Zou et al. 2012; Gomez-Coronado et al. 2016). Thirdly, nitrogen (N), one of the major agricultural practices in crop production, appears to be a promising agronomic strategy for the biofortification with Zn. Some studies showed that adequate N supply could effectively enhance the accumulation of Zn in wheat grains (Xue et al. 2012; Guo et al. 2013). This effect is dependent on sufficient Zn availability and enhanced by high Zn supply (Kutman et al. 2011; Guo et al. 2013). This beneficial effect is related to the fact that the supply of enough amounts of N increases the grain protein contents, which are positively correlated with grain Zn content (Zhao et al. 2009; Erenoglu et al. 2011).

Most of the studies assessing the relation between N nutrition and Zn accumulation in grains were carried out in hydroponics or greenhouse conditions and normally using a

single genotype. In fact, little information is available about the combined effects on different bread wheat genotypes of the combined foliar application of N and Zn. Moreover, to establish if the combined application of urea with one Zn foliar treatment could replace the traditional two Zn foliar applications could be economically interested, because Zn fertilizer is more expensive than urea (US$ 500 vs. US$ 300). Therefore, the aim of this study was to evaluate the genetic potential of four advanced breeding lines and two commercial varieties of bread wheat to different Zn and N fertilization treatments in terms of grain Zn accumulation under Mediterranean conditions. The effect on grain yield, yield components and grain quality was also evaluated.

Materials and Methods

A field experiment was conducted in Elvas, southern Portugal (38°53'N, 7°2'W, 186 m above sea level), in a Xerofluvents soil under rainfed Mediterranean conditions in 2012–2013 growing season. The mean temperature of the studied growing season was 17.3 °C, being the average temperature in the coldest month (January) and in the hottest month (July) 8.9 °C and 26.5 °C, respectively. Rainfall during the growing period (from late December to July) was 372 mm. All climate data were taken from a weather station located at the study site.

The experiment was arranged in a split plot design with three replications. Main plots were Zn treatments: i) non-Zn application (Control), ii) two foliar Zn applications (Foliar), iii) one foliar Zn with N application (Foliar + N), iv) soil and two foliar Zn applications (Soil + Foliar) and v) soil and one foliar Zn with N application (Soil + Foliar + N). Subplots were genotypes: four advanced lines of spring wheat from the Portuguese Institute of Agricultural and Veterinary Research (INIAV-1, INIAV-2, INIAV-3 and INIAV-4) (Table 1) and two Portuguese commercial varieties (Nabao and Roxo) currently cultivated by farmers which were chosen as controls. All these genotypes were chosen from the previous study of Gomez-Coronado et al. (2016) because their higher efficiency in grain Zn accumulation. Foliar Zn treatment consisted on the spray of 0.5% (w/v) of aqueous solution of $ZnSO_4 \cdot 7H_2O$ ha^{-1} with 800 L per hectare sprayed as described by Cakmak et al. (2010). Foliar and Soil + Foliar treatments were applied at anthesis and grain milk stage; Foliar + N and Soil + Foliar + N treatments only between treatments and at the anthesis stage. Soil treatment consisted on the spray to the soil surface and then incorpo-

Table 1. Pedigree details of the four advanced lines of spring wheat from the Portuguese Wheat Breeding Program (Portuguese Institute of Agricultural and Veterinary Research)

Name	Advanced line
INIAV-1	F900K/PRINIA
INIAV-2	SUNCO/2*PASTOR
INIAV-3	PRL/2*PASTOR
INIAV-4	CHEN/AEGILOPS-SQUARROSA (TAUS)//BCN/3/BAV92

rated into the soil before sowing of 50 kg $ZnSO_4 \cdot 7H_2O$ ha^{-1}. Nitrogen was applied as urea (46%) at a dose of 4 kg N ha^{-1} together with the Zn treatment at anthesis stage. An N-P-K fertilizer (15-15-15) was applied before sowing at a 200 kg ha^{-1} dose in all the plots and 100 kg N ha^{-1} as urea (56%) was applied at the beginning of tillering. The dose and form of the N-P-K fertilizer application was based on the local common crop management. The harvested area for each treatment was 4.2 m^2 (1.2 m × 3.5 m) with a space of 0.4 m between elementary plots. Land area used for experimental plots had not been previously fertilized with Zn, therefore a potential residual effect of any of them in the soil can be ruled out. The sowing was in late December, at a rate of 350 seeds m^{-2}.

Four soil samples of 30 cm depth were analysed for plant-available Zn, determined according to Lindsay and Norvell (1978) by extraction with DTPA (diethylenetriamine pentaacetic acid) and then determined by inductively-coupled plasma optical emission spectroscopy (ICP-OES, Vista-Pro Axial, Varian Pty Ltd, Mulgrave, Australia).

Harvesting was at maturity in early July. Grain yield (expressed as kg ha^{-1}), thousand-grain weight, test weight, grain protein content and total grain Zn content were determined. Nitrogen content was determined using the Dumas combustion method (Leco FP-428 analyser). Grain protein was determined by multiplying the total N by 5.7 as a conversion factor. Total grain Zn content was determined, after grain hand-threshed and digested with a mix of nitric acid and hydrogen peroxide using a closed-vessel microwave accelerated reaction system (CEM Corp, Matthews, USA). Contents of total Zn were measured by ICP-OES. Zinc uptake was calculated multiplying grain yield by total Zn in grain (expressed as g ha^{-1}). To estimate grain Zn bioavailability, phytate content was determined. The phytate assay was based on precipitation of ferric phytate and measurement of iron (Fe) remaining in the supernatant (Haug and Lantzsch 1983). Phytate was extracted from about 0.2 g of ground bread wheat grains, and the light absorbance was measured with a spectrophotometer at 419 nm (Gomez-Coronado et al. 2016). The molar ratio between phytate and Zn was calculated.

Data on grain yield, thousand-grain weight, test weight, grain protein content, total Zn content, Zn uptake and phytate:Zn ratio were subjected to a two-way ANOVA, including the Zn treatment (Control, Foliar, Foliar + N, Soil + Foliar and Soil + Foliar + N), cultivar (INIAV-1, INIAV-2, INIAV-3, INIAV-4, Nabao and Roxo) and their interaction in the model. When significant differences were found in ANOVA, means were compared using Fisher's protected least significant difference (LSD) test at $P \leq 0.05$. All analyses were performed using Statistic v. 8.10 for Windows (Analytical Software, Tallahassee, FL, USA).

Results

The initial analysis of the soil of the study showed that the DTPA-extractable Zn was 0.28 ± 0.02 mg kg^{-1} (mean ± standard error). The grain yield, thousand-grain weight and test weight were significantly affected ($p \leq 0.05$) by the Zn treatment, cultivar and by their interaction (except test weight) (Table 2). Significant differences were recorded between the different studied cultivars in the non-fertilized plots, ranging from 2653 kg ha^{-1} to

Table 2. Two-way ANOVA showing the effect of Zn treatment, cultivar and their interaction on each parameter evaluated (grain yield, thousand-grain weight, test weight, grain protein, total Zn content, Zn uptake and phytate: Zn ratio)

	DF	Grain yield *F*	Thousand-grain weight *F*	Test weight *F*	Grain protein *F*
Zn treatment	4	11.1***	195.4***	82.9***	41.1***
Cultivar	4	8.0***	19.1***	3.0*	6.1***
Zn treatment*Cultivar	20	2.6**	3.4***	1.5	3.0***
	DF	Total Zn content *F*	Zn uptake *F*	Phytate: Zn ratio *F*	
Zn treatment	4	12.5***	5.7***	8.4***	
Cultivar	4	471.4***	212.4***	357.7***	
Zn treatment*Cultivar	20	1.8*	2.5**	3.5***	

DF – degree of freedom; *F* values, including the level of significance (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$) are shown in the rest of the rows.

3841 kg ha^{-1}. Soil Zn treatments influenced positively on grain yield in both treatments, Soil + Foliar and Soil + Foliar + N, with average increases respecting control plots of 212 and 350 kg ha^{-1}, respectively. INIAV-2 and Nabao were, excepting in Soil + Foliar and Soil + Foliar + N treatments the less productive cultivars. INIAV-1, on the other hand, highlights as the most productive cultivar in all the treatments (Table 3). Regarding grain yield components, the inclusion of N in the treatments produced the lowest values of both, thousand-grain weight and test weight, with values lower than 38 g and 83 kg Hl^{-1}, respectively. INIAV-4 was the advanced line with higher thousand-grain weight in all Zn treatments and Nabao that with the lowest. Roxo was significantly the cultivar with higher test weight, with 85 kg Hl^{-1}, and INIAV-3 and INIAV-4 showed the lowest with 81 and 82 kg Hl^{-1}, respectively (Table 3).

Protein content was significantly influenced ($p \leq 0.001$) by the Zn treatment, cultivar and their interaction (Table 2), being significantly higher in the treatments with higher applications of Zn, i.e. Foliar and Soil + Foliar. Roxo, in all the Zn treatments (except in Soil + Foliar + N) was the one with the highest protein contents, and INIAV-2 and INIAV-4 obtained, on average, the lowest contents (Table 3).

Total content of Zn in the grain and Zn uptake were significantly affected ($p \leq 0.05$) by the Zn treatment, wheat cultivar as well as by their interaction (Table 2). Total Zn content in Control plots ranged between 14.0 and 20.3 mg kg^{-1} and Zn uptake between 44 and 67 g ha^{-1} (Table 3). However, in comparison with the Control treatment, the combined application of Zn and N, increased grain Zn contents up to 31 mg kg^{-1} in Foliar + N treatment and up to 31.8 mg kg^{-1} in Soil + Foliar + N treatment. The increments were even higher when two Zn foliar treatments were applied, up to 48 mg kg^{-1} in both, Foliar and Soil + Foliar treatments, highlighting INIAV-1 (in Foliar treatment) and the commercial varieties Nabao and Roxo. Regarding Zn uptake, the significant sequence was: Soil +

Table 3. Mean ± standard error in grain yield (kg ha^{-1}), thousand-grain weight (g), grain protein (%), total Zn content (mg kg^{-1}), Zn uptake (g ha^{-1}) and phytate:Zn molar ratio as affected by Zn treatment and cultivar

Treatment	Cultivar	Zn treatment					
		Control	Foliar + N	Foliar	Soil + Foliar + N	Soil + Foliar	Mean
Grain yield (kg ha^{-1})	INIAV-1	3702 ± 26 abcd	3512 ± 210 abcdef	3659 ± 56 abcde	3743 ± 121 abc	3480 ± 153 abcdef	3619 ± 70 α
	INIAV-2	2653 ± 158 i	3395 ± 70 bcdefg	3208 ± 55 efgh	3734 ± 108 abc	3592 ± 216 abcdef	3298 ± 131 β
	INIAV-3	3739 ± 33 abc	3755 ± 109 abc	3465 ± 181 bcdef	3733 ± 176 abcd	3564 ± 163 abcdef	3651 ± 87 α
	INIAV-4	3841 ± 174 ab	3478 ± 285 abcdef	2952 ± 104 ghi	3579 ± 244 abcdef	3695 ± 133 abcd	3509 ± 139 α
	Nabao	2873 ± 150 hi	2673 ± 136 i	2667 ± 151 i	3355 ± 57 cdefg	3389 ± 161 bcdefg	2992 ± 107 γ
	Roxo	3266 ± 160 defgh	3416 ± 140 bcdefg	3187 ± 171 fgh	3938 ± 200 a	3539 ± 189 abcdef	3469± 100 αβ
Thousand-grain weight (g)	INIAV-1	41 ± 2.8 fghi	39 ±2.7 ghijk	43 ± 4.1 cde	41 ± 4.0 efghi	42 ± 3.8 def	41 ± 2.3 β
	INIAV-2	38 ± 2.8 ijkl	38 ± 3.2 klm	39 ± 3.1 hijkl	38 ± 3.4 jklm	39 ± 3.6 jklm	39 ± 3.5 π
	INIAV-3	41 ± 3.8 efgh	37 ± 3.6 lm	45 ± 4.8 ab	37 ± 2.1 m	43 ± 4.1 bcd	41 ± 3.2 β
	INIAV-4	46 ± 4.3 a	44 ± 3.2 abc	45 ± 4.2 ab	45 ± 4.8 ab	45 ± 4.7 abc	45 ± 4.2 α
	Nabao	33 ± 2.7 n	29 ± 1.2 p	31 ± 2.9 no	30 ± 2.7 op	32 ± 3.0 no	31 ± 1.8 σ
	Roxo	40 ± 3.2 fghij	38 ± 3.2 klm	41 ± 2.7 efg	38 ± 1.8 jklm	40 ± 3.6 ghijk	40 ± 3.8 γ
Grain protein (%)	INIAV-1	9.3 ± 0.6 ghijkl	9.5 ± 0.9 fghijk	9.8 ± 0.7 defgh	10.3 ± 0.9 abcd	9.7 ± 0.9 defghi	9.7 ± 0.3 β
	INIAV-2	8.5 ± 0.5 n	9.1 ± 0.7 jklmn	8.7 ± 0.8 mn	8.9 ± 0.8 lmn	9.4 ± 0.8 ghijkl	8.9 ± 0.5 π
	INIAV-3	9.1 ± 0.7 ijklmn	8.9 ± 0.8 klmn	9.3 ± 0.8 hijklm	9.3 ± 0.8 ghijklm	9.9 ± 0.9 cdefg	9.3 ± 0.8 γ
	INIAV-4	8.9 ± 0.7 klmn	8.8 ± 0.8 lmn	9.5 ± 0.6 fghijk	8.5 ± 0.7 n	9.5 ± 0.7 fghijk	9.1 ± 0.2 γπ
	Nabao	10.2 ± 1.1 bcde	9.6 ± 0.8 efghij	10.6 ± 0.9 ab	9.0 ± 0.8 jklmn	10.0 ± 0.9 cdef	9.9 ± 1.0 β
	Roxo	10.7 ± 0.8 ab	10.6 ± 0.9 ab	10.5 ± 0.9 ab	10.2 ± 1.1 bcd	10.9 ± 1.1 a	10.6 ± 1.5 α

Table 3 (cont.)

Treatment	Cultivar	Zn treatment					
		Control	Foliar + N	Foliar	Soil + Foliar + N	Soil + Foliar	Mean
Total Zn content (mg kg^{-1})	INIAV-1	14.0 ± 2.0 k	31.0 ± 2.8 gh	50.7 ± 3.5 abcd	31.3 ± 2.4 gh	47.7 ± 2.7 bcde	34.9 ± 3.2 β
	INIAV-2	17.0 ± 1.3 ijk	31.7 ± 2.7 gh	45.7 ± 4.8 e	28.3 ± 1.9 h	43.7 ± 3.3 e	33.3 ± 3.1 βγ
	INIAV-3	16.0 ± 1.1 jk	28.3 ± 1.8 h	44.0 ± 4.3 e	31.0 ± 1.5 gh	45.0 ± 2.8 e	32.9 ± 3.0 γ
	INIAV-4	17.3 ± 1.7 ijk	29.7 ± 2.6 gh	47.3 ± 2.8 cde	29.7 ± 4.0 gh	46.7 ± 4.4 de	34.1 ± 3.5 βγ
	Nabao	20.0 ± 2.1 ij	32.0 ± 2.3 gh	50.7 ± 3.9 abcd	32.7 ± 2.1 g	54.0 ± 6.0 a	37.9 ± 2.9 α
	Roxo	20.3 ± 1.0 i	31.0 ± 3.1 gh	51.7 ± 1.6 ab	37.7 ± 2.9 f	51.0 ± 1.9 abc	38.3 ± 4.0 α
Zn uptake (g ha^{-1})	INIAV-1	52 ± 4.7 kl	110 ± 10.4 h	185 ± 6.1 a	117 ± 5.9 gh	166 ± 16.1 abcd	126 ± 12.5 αβ
	INIAV-2	44 ± 5.0 l	108 ± 7.9 h	147 ± 15.0 def	106 ± 4.8 hi	157 ± 16.2 cde	112 ± 10.9 γ
	INIAV-3	59 ± 2.9 kl	106 ± 3.6 hi	151 ± 2.9 def	116 ± 11.1 gh	161 ± 11.3 bcde	119 ± 11.5 βγ
	INIAV-4	67 ± 6.7 jk	103 ± 9.2 hi	140 ± 12.2 ef	116 ± 11.2 hi	172 ± 17.2 abc	118 ± 11.2 βγ
	Nabao	57 ± 6.0 kl	86 ± 8.2 ij	135 ± 11.0 fg	110 ± 6.9 h	182 ± 18.1 a	114 ± 11.3 γ
	Roxo	67 ± 4.8 jk	106 ± 10.6 hi	165 ± 12 abcd	148 ± 5.2 def	180 ± 6.2 ab	133 ± 13.1 α
Phytate:Zn ratio	INIAV-1	52.8 ± 4.2 a	23.5 ± 2.3 d	14.3 ± 1.3 g	23.1 ± 2.1 de	15.0 ± 1.0 fg	25.8 ± 2.8 α
	INIAV-2	43.3 ± 4.1 b	23.0 ± 2.2 de	15.9 ± 1.4 fg	25.5 ± 1.6 d	16.6 ± 1.3 fg	24.9 ± 3.0 α
	INIAV-3	45.3 ± 3.1 b	25.6 ± 1.9 d	16.6 ± 2.4 fg	23.4 ± 1.5 d	16.1 ± 1.2 fg	25.4 ± 2.6 α
	INIAV-4	42.0 ± 2.1 b	24.5 ± 1.4 d	15.3 ± 2.0 fg	24.4 ± 2.4 d	15.9 ± 2.0 fg	24.3 ± 2.1 α
	Nabao	36.6 ± 2.9 c	22.7 ± 2.4 de	14.3 ± 1.3 g	22.2 ± 2.1 de	13.5 ± 1.4 g	21.9 ± 3.0 β
	Roxo	35.6 ± 1.8 c	23.4 ± 3.0 de	14.0 ± 1.3 g	19.3 ± 2.0 ef	14.2 ± 1.4 g	21.3 ± 2.0 β

For each parameter, averages in the Mean column with different Greek letter mean significant effect of cultivar ($p \leq 0.05$) according to LSD test; averages with different lowercase letter are significantly affected by cultivar*Zn treatment ($p \leq 0.05$) according to LSD test.

Foliar > Foliar > Soil + Foliar + N > Foliar + N > Control, with 170, 154, 117, 103 and 58 g ha^{-1}, respectively (Table 3). Regarding Zn uptake, INIAV-1 and Roxo were significantly the most efficient cultivars in the average treatment as well as in the Foliar treatment (Table 3).

Phytate:Zn molar ratio was significantly affected by the Zn treatment, cultivar and their interaction (Table 2). The lowest phytate:Zn ratios were obtained when two foliar Zn applications were carried out, i.e. Foliar and Soil + Foliar treatments, being INIAV-1, Nabao and Roxo those with ratios lower than 15. The combined application of Zn with N, in both treatments, resulted in significantly higher ratios, with average values higher than 23.0. The highest ratios were obtained in the non-fertilized plots, being INIAV-1 that with the highest ratio, 52.8 (Table 3).

Discussion

Under soil deficient Zn-contents, as in this study with 0.28 mg kg^{-1} of DTPA-Zn, plant breeding depends completely on application of Zn-containing fertilizers (Alloway 2009; Bouis and Welch 2010; Joy et al. 2015; Gomez-Coronado et al. 2016). Any of the studied genotypes, although in consonance with Zn contents found by Terrés et al. (2001) and Galinha et al. (2013) in Portugal, reached the target level of 38 mg Zn kg^{-1} established in the Harvestplus program neither the phytate:Zn ratios lower than 15 (Gargari et al. 2007). However, the variability found between the different cultivars was wide enough to ensure the development of a successful genetic biofortification program, being Nabao and Roxo the varieties with better contents and bioavailability (Table 3). Regarding grain yield, considered another key target to achieve, variation was also wide between cultivars (in more than 1100 kg ha^{-1}) being INIAV-1, INIAV-3 and INIAV-4 the most productive genotypes in this Zn deficient soil. Because Zn uptake is the result of multiplying grain yield by total Zn content in grain, INIAV-4 and Roxo were the cultivars with higher accumulation of Zn per ha, with more than 67 g Zn ha^{-1} (Table 3). Regarding quality, Roxo highlighted again with higher contents in grain protein.

Based on the reports published by Graham et al. (2007) and Cakmak et al. (2010) in wheat, establishing that an agronomic biofortification practice could be considered with a measurable biological impact on human health when produced an increase of at least 10 mg Zn kg^{-1}, all the treatments reported were successful. However, the target level established by the Harvestplus program or Wang et al. (2012) only was reached when two foliar Zn applications were applied, i.e. Foliar and Soil + Foliar treatments. Foliar application, in both Foliar and Soil + Foliar treatments, produced increases in Zn contents of about 2.8 times respecting non-fertilized plots, achieving an average concentration higher than 48.4 mg Zn kg^{-1}. Lower increases were found by Zhang et al. (2012), of about 26–115% and 68%, respectively, with foliar applications, or by Zou et al. (2012), of about 83.5% with Soil + Foliar applications but similar with Gomez-Coronado et al. (2016) in the same region. However, when one foliar Zn application was substituted by urea, Zn contents was on average 31–32 mg kg^{-1}, supposing an increase of more than 1.8 times with respect to non-fertilized plots, but without reaching the target levels in any case

(Table 3). Therefore, the substitution of one foliar Zn treatment during grain filling by the application of 4 kg ha^{-1} of urea combined with the Zn application at anthesis, in contrast of our initial hypothesis was not enough to achieve levels higher than the recommended.

An adequate Zn bioavailability, established by Gargari et al. (2007) and Pfeiffer and McClafferty (2007) by phytate:Zn molar ratio lower than 15 was only achieved on INIAV-1, Nabao and Roxo when two foliar Zn applications were applied. This meant a reduction of about 182% compared to the Control, and about 55% regarding the combined application of Zn and urea. Wang et al. (2015) also found significant decreases in phytate:Zn ratios with soil applications of Zn. Supposing the intake of 100 g bread made with these whole grains treated with Foliar or Soil + Foliar treatments may provided about 4 to 4.8 mg Zn, about one third of the Zn RDI for humans, and about 2.7 times more than the same intake of non-biofortified wheat. Moreover, the advanced line INIAV-1 and both commercial varieties should be taking into account for future Zn biofortification programs. Conversely, INIAV-3 and INIAV-4 accumulate less Zn in the grain in every Zn treatment so, from biofortification point of view, they must be discarded (Table 3).

The implementation of a biofortification program which increases grain yield could be really successful being the combination of genetic and agronomic biofortification the best option. As expected, soil application, in both treatments, Soil + Foliar and Soil + Foliar + N, produced the highest grain yields, which were on average about 10% higher with respect to non-fertilised plots or Zn foliar treatments (Table 3). Zou et al. (2012) obtained increases even lower, in a research conducted in 14 locations from seven countries, with an average increase of 5.1%, but more similar to the increases obtained for them in Pakistan (average increase of 13.7%). This might be related to the low DTPA-Zn levels in soil, indicating that Zn deficiency was a growth-limiting factor on the experimental soils as Cakmak et al. (2010) and Gomez-Coronado et al. (2016) found in soils with less of 0.3 mg DTPA-Zn kg^{-1}. Foliar Zn application had not any positive effect on grain yield, in contrast of Karim et al. (2012) who found an improvement on growth and antioxidative defence mechanisms of plants against drought-induced oxidative cell damage under drought conditions with foliar Zn sprays but according with Cakmak et al. (2010), Gomez-Coronado et al. (2016) and Guo et al. (2016). The higher grain yield found in both Soil + Foliar treatments and in some cultivars could produce a possible dilution effect. To avoid it, Zn uptake expressed in g Zn ha^{-1} was determined resulting in higher uptakes in the treatments with higher Zn levels, with more than 40% with respect to their respective treatment without N. This positive effect is attributed to a growth enhancement (Aciksoz et al. 2011) and to the positive effects on improving root Zn uptake (Erenoglu et al. 2011; Kutman et al. 2012). Although N supply has a clear effect on senescence, plants remained green longer and having longer grain-filling periods (Kutman et al. 2011), a higher Zn application was more effective in the Zn grain accumulation. Regarding the genotypic variation, INIAV-1 must be highly featured because it highlights in all the studied Zn treatments in grain Zn content, Zn uptake and grain yield (Table 3). On further research, studies should be done combining urea and Zn in different doses and genotypes to determine their interaction.

Grain protein content is a characteristic with a marked genetic load. Nevertheless, the application of Zn fertilizer at grain filling stage improved significantly it (Table 3). It could be due to the close link found by Cakmak et al. (2004) and Ghasemi et al. (2013) between the genes affecting the grain Zn accumulation and protein content in *Triticum dicoccoides*. This indicates that Zn and protein contents might have the same genetic base to some extent, and could be simultaneously improved by breeding (Welch and Graham 2002). Commercial variety Roxo and the cultivar INIAV-1 were, once more, the ones with the best quality.

The fact that none of the Zn applications had negative effects neither grain yield nor quality (even Soil + Foliar treatment increased moderately grain yield) could be considered as very positive point. It would be very difficult to successfully implement a biofortification program if the farmers´ income was lower as a consequence of the Zn application. Regarding the costs, and taking into account only the fertilizers, if one ton of $ZnSO_4 \cdot 7H_2O$ is about US$ 500 (50 kg ha^{-1} of soil application is about US$ 25; and 4 kg ha^{-1} of each foliar application is US$ 2) and one ton of urea is about US$ 300 (4 kg ha^{-1} of foliar application is US$ 1.2), costs would be from US$ 3.2 to US$ 4 in Foliar + N and Foliar treatments up to US$ 30.2 to US$ 33 in Soil + Foliar + N and Soil + Foliar treatments, respectively. In both treatments including soil application, the extra cost would be almost covered by the increase of grain yield, being this application economically interesting from the farmers' point of view. On the other hand, the extra cost of the foliar application should be assumed either by the Authorities or by the consumers.

The present study shows the strong influence of the genetic load on the grain Zn accumulation. However, due to the deficient level of available Zn in the soil, the grain Zn contents were not enough to achieve the target level established on 38 mg Zn kg^{-1}, being completely necessary to complement the genetic biofortification with the agronomic biofortification. Only with the double foliar Zn applications (Foliar and Soil + Foliar treatments) the target level with an adequate bioavailability were achieved. The replacement of the second foliar Zn application by 4 kg urea ha^{-1} did not produce sufficient Zn content increases neither the sufficient bioavailability. Soil Zn application produced in both treatments, Soil + Foliar and Soil + Foliar + N, grain yield increases between 7–10%, which virtually offset the extra expense of the application. The most interesting cultivars in all the treatments, even in the non-fertilized plots, were INIAV-1, Nabao and Roxo being the best options to biofortify with the Soil + Foliar treatment to establish a program of biofortification in Zn-deficient soils.

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