An Evaluation of Kernel Zinc in Hybrids of Elite Quality Protein Maize (QPM) and Non-QPM Inbred Lines Adapted to the Tropics Based on a Mating Design

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Abstract: Genetic improvement of maize with elevated levels of zinc (Zn) can reduce Zn deficiency among populations who rely on maize as a staple. Inbred lines of quality protein maize (QPM) and non-QPM with elevated Zn levels in the kernel have been identified. However, information about the optimal strategy to utilize the germplasm in breeding for high-Zn concentration is lacking. As a preliminary step, this study was conducted to ascertain the potential of QPM, non-QPM, or a combination of QPM and non-QPM hybrids for attaining desirable Zn concentration. Twenty elite inbreds, 10 QPM and 10 non-QPM, were crossed according to a modified mating design to generate hybrids, which were evaluated in four environments in Mexico during 2015 and 2016 in order to evaluate their merits as parents of hybrids. The highest mean values of Zn were observed when high-Zn QPM lines were crossed with high-Zn non-QPM lines. Hybrids with high Zn and grain yield were identified. General combining ability (GCA) effects for Zn concentration were more preponderant than specific combining ability (SCA) effects, suggesting the importance of additive gene action for the inheritance of Zn.

Keywords: genetics; maize; zinc; QPM; kernels; combining ability; breeding

1. Introduction

Micronutrient deficiency, resulting from inadequate intake of essential minerals such as zinc (Zn), is an increasingly serious food-related health problem [1]. Approximately 20% of the world's population suffers from Zn deficiency, with the highest risks for young children and pregnant women in sub-Saharan Africa and South Asia [2]. Approaches to mitigate Zn deficiency include diet supplementation, industrial fortification, and food diversification. However, at a large scale, the impact of these interventions remains limited, especially in low-income countries, due to recurrent costs, poor infrastructure, and delivery systems [3,4]. Therefore, development of Zn-enriched staple crops through breeding may complement those options [5–7].

Maize is one of the major crops grown and consumed in regions where Zn deficiency is prevalent [8–10]. For instance, in sub-Saharan Africa, 80% of the maize is consumed directly as food, providing at least 30% of the total calories [10–12]. However, maize improvement programs have primarily focused on developing high-yielding varieties able to tolerate various biotic and abiotic

stress factors in different agro-ecologies [13]. Therefore, the production of micronutrient-rich varieties has lagged behind the improvement of other traits.

The physiological processes by which Zn accumulates in the maize kernels have not been completely described. A maize plant acquires Zn through the roots with uptake mediated by Zn-regulated transporters [14]. Then, Zn is transferred to the vascular bundles for transport to the shoot [15] and remobilized from the leaves into the kernels during grain-filling [16]. In kernels, higher concentrations of Zn are observed in the aleurone and embryo than in the endosperm [17–20].

The plant's ability to accumulate Zn in the kernels can also be influenced by environmental conditions and soil properties. For example, an increase in soil pH decreases the uptake of Zn from the soil and reduces its availability to the plant [21,22]. Low soil moisture, organic matter content, and temperature impairs Zn diffusion to the roots causing reductions in uptake and translocation into the shoot [23–25]. Consequently, the genetic capacity of a plant to absorb Zn from the soil and accumulate it in the kernels for optimal nutritional benefit may not be fully realized.

The successful identification of desirable hybrid combinations depends on the combining ability of the parents and the gene effects involved in the expression of a trait. Several genetic studies involving mating designs documented that for kernel Zn general combining ability (GCA) effects were greater than specific combining ability (SCA) [26–29]. Significant GCA effects indicate the preponderance of additive gene action for kernel Zn inheritance, implying that genetic gains can be realized from selection.

Kernel Zn has been investigated in several analyses of quantitative trait loci (QTL), which have shown that Zn accumulation is under the control of several loci, from 4 to 20 per population [30–35]. Additionally, genomic regions associated with important QTLs for kernel Zn have been reported on chromosomes 2 and 6 [34,35]. Consistent with mating designs, additive gene effects predominantly controlled kernel Zn concentration in the QTL studies. The QTL studies, however, were conducted with populations of inbred progeny created from parents unadapted to tropical environments [31–35]. So, the relevance of those studies for hybrid breeding for tropical environments may be limited.

In maize, nutritional-related research has emphasized quality protein maize (QPM) to address protein malnutrition [36–38]. QPM inbred lines are bred to be homozygous for a recessive allele at the opaque-2 locus, with elevated levels of amino acids lysine and tryptophan, and a hard endosperm in elite genetic backgrounds. Inbred lines with high-Zn have been identified among QPM [18,39–44]. The high-Zn values suggest a possible influence of opaque2 (*o2*) locus or possibly other genetic factors present in the QPM lines [44]. A key hypothesis of this study is that some genetic effects for increased levels of zinc in some QPM inbred lines will be observed in their hybrid progeny. However, QPM maize with relatively low levels of Zn have been observed, suggesting that although *o2* may play an important role, there might be other favorable loci unrelated to *o2* that are required for the enhancement of Zn [18,40].

Significant differences in concentration of Zn have also been documented among non-QPM inbred lines [28,39,43,45–49]. The variability for kernel Zn among the inbred lines suggests a possibility to enhance the Zn content in maize [4]. In the present study, groups of QPM (high-Zn QPM and low-Zn QPM) and non-QPM (high-Zn non-QPM and low-Zn non-QPM) inbred lines adapted to tropical environments were mated to produce hybrids using a modified mating design. The objectives of this study were (i) to estimate the combining ability of elite QPM and non-QPM inbred lines for kernel Zn, (ii) to explore the potential of developing high-Zn hybrids using QPM, non-QPM, and/or a combination of QPM and non-QPM inbred lines, (iii) to investigate the relationship between kernel Zn and other traits of agronomic importance, and (iv) to evaluate the relative importance of additive and non-additive genetic effects for Zn.

2. Materials and Methods

2.1. Plant Material

Ten quality protein maize (QPM) and 10 non-QPM inbred lines adapted to tropical and sub-tropical environments were selected for this study (Table 1). The inbred lines were selected based on the Zn level in the kernel and whether classified as QPM or non QPM.

The Zn levels of the inbreds, groups A–D, were based on evaluations in previous seasons in the same environments used for this study. In addition, the lines were developed and selected based on their agronomic performance and potential to serve as parents of hybrids suitable for production in the target environments in Central America and Mexico. The lines were divided into four groups of five inbreds, according to their Zn levels and whether classified as QPM or non-QPM, based on their lysine and tryptophan content. The four groups were high-Zn QPM (>33 μg/g, Zn target level for nutritional impact; [5]), low-Zn QPM (<33 μg/g), low-Zn non-QPM, and high- Zn non-QPM (Table 1). Intergroup crosses were made by mating each line from one group to the five lines in another group to form six sets of 25 hybrids (Table S2). Five of the six sets produced enough kernels for evaluation in trials from all 25 expected hybrids per set. Two crosses between inbred lines 13 and 16 and 15 and 17 in set six did not produce enough kernels, and the crosses were discarded. Therefore, set six produced 23 hybrids instead of 25. Kernels from reciprocal crosses in each set were bulked. In total, 148 hybrids were formed at Agua Fria, Puebla, Mexico, between November 2014 and May 2015.

The mating among the inbreds was intended to fulfill the requirements of a North Carolina design II [50]. However, mating among the lines in each set was slightly different from the standard design II [50], since each inbred line was not used strictly as female or male (Table S1). All inbred lines were used multiple times (as females, males, or both) to form hybrids in different sets (Table 1). As a consequence, variance components (σ_A and σ_D) could not be estimated since: (i) the inbred lines were selections made from a breeding program, and not a random sample from a population, (ii) for each set, the sample size was small (10 inbred lines per set), and (iii) data could not be pooled across sets to estimate the sum of squares because the same inbred lines were used to form hybrids in different sets. Hence, analyses were conducted within sets to calculate means for the hybrids and estimates of GCA and SCA effects of inbred lines and hybrids, respectively. These parameters are not influenced by the variance components.

A total of 148 hybrids plus two commercial hybrid checks were grown in four environments at the research stations of CIMMYT (International Maize and Wheat Improvement Center) and INIFAP (National Agricultural Research Institute) in Mexico, during the months of June through October. The four environments were Tlaltizapan (18°41′ N, 99°07′ W; 962.5 m above sea level (m asl)) during 2015 and 2016; Agua Fria (20°32′ N, 97°28′ W, 110 m asl) during 2015; and Cotaxtla (18°49′ N, 96°22′ W, 57 m a.s.l.) during 2015. The experimental design was an alpha-lattice [51] using two replications and one-row plots. All plots were managed according to the recommended agronomic practices for each environment.

Table 1. List of 20 elite maize inbred lines used as parents for the mating design.

Line	Pedigree	Group	Description	Role of Inbred Line	Set
1	((CML491/LAPOSTASEQ-C7-F64-2-6-2-2-B*3)/CML491)-B-7-1-1-1-1-B	A	High Zn, QPM	Female	1, 2 & 3
2	((CML491/LAPOSTASEQ-C7-F64-2-6-2-2-B*3)/CML491)-B-37-1-1-1-1-B	A	High Zn, QPM	Female	1, 2 & 3
3	((CML491/(((((CML176/R1)/R1)-73-1-3-1/R1)-79/R1)-36-1-B)/CML491)-B-18-1-1-1-1-B	A	High Zn, QPM	Female	1, 2 & 3
4	((CML491/LAPOSTASEQ-C7-F103-2-2-2-1-B*3)/CML491)-B-17-2-1-1-1-1-B	A	High Zn, QPM	Female	1, 2 & 3
5	((CML491/LAPOSTASEQ-C7-F64-2-6-2-1-B-B)/CML491)-B-50-1-2-1-1-B	A	High Zn, QPM	Female	1, 2 & 3
6	((CML491/CML150)/CML491)-B-13-1-1-1-1-B-B	B	Low Zn, QPM	Male & Female	1, 4 & 5
7	((CML491/CML150)/CML491)-B-21-1-1-1-1-B-B	B	Low Zn, QPM	Male & Female	1, 4 & 5
8	((CML491/LAPOSTASEQ-C7-F64-2-6-2-1-B-B)/CML491)-B-30-1-1-1-1-B-B	B	Low Zn, QPM	Male & Female	1, 4 & 5
9	CML247Q	B	Low Zn, QPM	Male & Female	1, 4 & 5
10	CML254Q	B	Low Zn, QPM	Male & Female	1, 4 & 5
11	(CML550/CML511)-B-62-2-1-1-B	C	Low Zn, Non-QPM	Male & Female	2, 4 & 6
12	(CLG2312/CML9)-B-80-1-1-1-B	C	Low Zn, Non-QPM	Male & Female	2, 4 & 6
13	(CML550/CML511)-B-106-1-1-1-B	C	Low Zn, Non-QPM	Male & Female	2, 4 & 6
14	((CRIOLLOTTH/CML247)/CLRCW105)-B-37-1-1-1-B	C	Low Zn, Non-QPM	Male & Female	2, 4 & 6
15	(CLG2312/CML505)-B-43-1-1-1-B	C	Low Zn, Non-QPM	Male & Female	2, 4 & 6
16	((CML247/(((((CML176/R1)/R1)-73-1-3-1/R1)-79/R1)-36-1-B)/CML247)-B-14-2-1-1-1-B-B	D	High Zn, Non-QPM	Male	3, 5 & 6
17	((CML247/(((((CML176/R1)/R1)-73-1-3-1/R1)-79/R1)-36-1-B)/CML247)-B-18-1-1-1-1-B-B	D	High Zn, Non-QPM	Male	3, 5 & 6
18	(CLRCW79/CLRCW98)-B-14-2-1-1-B-B	D	High Zn, Non-QPM	Male	3, 5 & 6
19	(CLRCW79/CLRCW98)-B-16-2-1-1-B-B	D	High Zn, Non-QPM	Male	3, 5 & 6
20	(CLRCW79/CLRCW98)-B-22-3-1-1-B-B	D	High Zn, Non-QPM	Male	3, 5 & 6

Zn = zinc, QPM = Quality Protein Maize.

2.2. Phenotypic Analysis

At Tlaltizapan, the experimental unit was a 5-m-long plot, with an inter-row spacing of 0.75 m and a spacing of 0.14 m between hills, giving a final plant density of approximately 93,000 plants ha^{-1}. At Agua Fria, the experimental unit was a 4.5-m-long plot, with an inter-row spacing of 0.75 m and a spacing of 0.30 m between hills, giving a final plant density of approximately 44,444 plants ha^{-1}, and at Cotaxtla the experimental unit was a 5-m-long plot, with an inter-row spacing of 0.80 m and a spacing of 0.20 m between hills, giving a final plant density of approximately 63,000 plants ha^{-1}.

Data were recorded on a plot basis on several traits in each experiment: days to anthesis, days to silking, anthesis silking interval (ASI), plant height, grain yield, and kernel Zn concentration. Days to anthesis was recorded as the number of days from planting to when 50% of the plants in a plot were shedding pollen, and days to silking was the number of days from planting to when 50% of the plants in a plot had extruded silks. ASI was determined as the difference between days to silking and days to anthesis. Plant height was measured in centimeters as the distance from the base of the plant to the top of the first tassel branch. Grain yield, expressed in tons ha^{-1}, was determined by adjusting the shelling and grain moisture percentages to 80% and 12.5%, respectively. Shelling percentage, calculated as ((grain weight/ear weight) × 100), was used to determine the grain weight in a plot. Normally, at harvest, the shelling percentage averages about 80% [52]. Grain moisture at harvest was measured using a hand-held moisture meter. Samples for grain moisture content were obtained by removing several rows of maize kernels from 10 randomly selected ears per plot/row.

The following formula was used to estimate grain yield in ton ha^{-1}:

$$\frac{field\ weight\ in\ kgs}{1000} \times \frac{100 - moisture\ content\ per\ plot}{100 - 12.5} \times \frac{10000}{plot\ area} \times shelling\ percentage$$

In each plot of all environments, four to six plants were self-pollinated and harvested with husk for the determination of kernel Zn concentration. These ears were not used to estimate grain yield.

At harvest, the self-pollinated ears from each plot were manually harvested and dried to a moisture content of 12.5%. Kernels from the ears were hand-shelled and bulked, and a representative sample was obtained from each plot. Approximately 50 g of kernels from each plot were ground to a fine powder (flour approx. 0.5 μm) using a Retsch™ miller (model MM400) and a 35 mL grinding milling jar of zirconium. Flour was collected in 15 mL plastic tubes for Zn content analysis using a 'bench-top', non-destructive, energy-dispersive X-ray fluorometer (XRF; Oxford instruments ™, model X-Supreme 8000®). Six grams of flour were placed into the polypropylene capsules and sealed with a Poly-4® XRF film for scanning. Briefly, before the samples were analyzed, the equipment was calibrated by relating the X-ray emission intensity of Zn to a group of samples whose Zn concentration had been previously determined through inductively coupled plasma optical emission spectrophotometer (ICP-OES). The calibrations were validated by comparing the values given by the XRF with those from the ICP-OES. This was done using a group of samples different from those used in the calibration. To confirm the values obtained by XRF, 10% of the samples were re-analyzed by the ICP-OES [53]. In the ICP-OES analysis, aluminum was also monitored as an indicator of contamination [40].

2.3. Data Analysis

The trials were analyzed according to an alpha-lattice design using multi-environmental trial analysis with R (META-R) [54]. Variance components due to genotypes ($\sigma^2 G$), genotypes by environment ($\sigma^2 G \times E$) interactions, and residual errors ($\sigma^2 e$) were estimated from the analysis of variance (ANOVA). Replications and incomplete blocks within replications were considered random effects while genotypes (hybrids) and environments were considered fixed effects. Genotypes were considered as fixed effects because they were developed from inbred lines that were specifically selected from a breeding program, had different levels of kernel Zn concentration, and were classified as QPM or non-QPM. Hence, inference was limited to the population from which the inbred line was selected from and to the

environments under which the hybrids were evaluated. Broad-sense heritability (H^2) for traits in individual and across environments was estimated using the variance components [55].

To estimate combining ability, a separate analysis of variance was conducted for the 148 hybrids (excluding the checks), according to a modified mating design using the proc GLM statement of SAS [56]. Hybrids were nested within sets for each environment and across environments. Components of variance due to hybrids within sets were divided into variance due to female (sets), male (sets), and the interaction between female × male (sets). The *F* tests for female (sets), male (sets), and female × male (sets) mean squares were computed using the mean squares for their respective interaction with environment. Mean squares attributable to female (sets) × environment, and male (sets) × environment were tested using the mean square for female × male (sets) × environment, whereas the mean square for female × male (sets) × environment was tested using the pooled error mean squares. The expectations of females (set) and males (set) represents the general combining ability (GCA_f and GCA_m) effects, while the interaction between female × male (set) represents specific combining ability (SCA) effects [55]. The proc mixed statement of SAS [56] was used to calculate adjusted means for grain yield and kernel Zn in individual sets. Sets were considered fixed effects; thus, their interpretation was based on means and differences, and inferences were limited only to the specified set [57]. The allocation of inbred lines as males or females was random. Therefore, males and females within sets, interaction of females and males within sets, and all their interactions with the environment were considered random effects. For random effects, the measure of interest is the variance [57], and inferences can be made relative to the reference population and how they interact with the environments [55]. Estimates of GCA effects for kernel Zn, grain yield, and days to flowering for the inbred lines were calculated in each environment and across environments. For kernel Zn concentration, the SCA effects for each cross over environments were also estimated.

Although mating among the lines deviated from a standard NC II, the expectations of the mean squares for females (set), males (set) and females × males (set) were the same for the components of variance and the covariances of relatives as in a standard NC II [55]. Assuming no epistasis and a coefficient of inbreeding of one, variance components can be expressed in terms of covariance (Cov) of relatives where $\sigma^2_{male} = \sigma^2_{female} = $ Cov half-sib $= (1/2)\ \sigma^2_A$, and $\sigma^2_{male \times female} = $ Cov full-sib − Cov half-sib male − Cov half-sib female $= \sigma^2_D$ [55]. Therefore, the variance explained by the GCA effects of parents = 1/2 additive genetic variance (VA) and the variance explained by SCA = dominance genetic variance (VD). The relative contribution of GCA (additive) and SCA (non-additive) genetic variances for kernel Zn in each set were computed relative to the total genetic variance.

3. Results

Genotypes (hybrids) and genotype × environment (G × E) variance components were significantly different ($p < 0.001$) for Zn and grain yield (Table 2). For Zn, the variance component for genotypes was larger (~3-fold) compared to the variance due to G × E and the heritability (H^2) estimate was 0.85. For grain yield, the variance due to genotypes was 5-fold larger than the G × E variance component and the H^2 estimate was 0.91. The heritability for grain yield, while numerically higher than that of Zn, is substantially equivalent. The estimates of heritability for both traits suggest environmental sources of variation were relatively low in this experiment.

Averages for Zn and grain yield of all hybrids for each environment and across environments were estimated (Table 2). The highest mean for Zn (26.51 μg/g) was observed in Tlaltizapan 2016 and the lowest was in Tlaltizapan 2015 (22.47 μg/g). Tlaltizapan 2016 was an exceptional environment in which 21 hybrids accumulated ≥30 μg/g of Zn (Table S3). The mean grain yield across environments was 7.07 t ha^{-1} with range of 8.75 t ha^{-1} for Tlaltizapan 2015 to 4.87 t ha^{-1} for Cotaxtla 2015 (Table 2).

Table 2. Performance of the top ranking 10% hybrids for Zn and their grain yield, averages, heritabilities, and variance components at each environment and across environments.

Hybrid	Cross	Group	Tlaltizapan 2015		Tlaltizapan 2016		Agua Fria 2015		Cotaxtla 2015		Average across Environments	
			GY	Zn	GY	Zn	GY	Zn	GY	Zn	GY	Zn
			t ha^{-1}	µg/g	t ha^{-1}	µg/g	t ha^{-1}	µg/g	t ha^{-1}	µg/g	t ha^{-1}	µg/g
56	2 × 16	A × D	10.35	28.32	9.14	28.96	6.04	32.94	6.79	32.58	8.22	31.45
51	1 × 16	A × D	9.73	26.78	11.07	31.35	7.74	33.33	5.80	30.46	8.80	31.07
60	2 × 20	A × D	11.18	25.80	9.51	32.76	8.93	35.38	7.31	25.79	9.40	30.25
57	2 × 17	A × D	9.80	25.27	10.12	30.20	5.92	31.90	4.71	28.21	7.80	29.26
55	1 × 20	A × D	10.01	25.39	11.24	29.69	7.70	32.80	4.90	25.61	8.61	29.07
1	1 × 6	A × B	6.20	26.85	6.34	30.10	2.88	31.34	2.31	24.22	4.11	28.72
21	5 × 6	A × B	3.84	27.83	4.72	31.50	1.79	26.43	1.37	-	2.48	28.66
23	5 × 8	A × B	5.95	26.06	5.60	32.25	4.60	25.42	2.91	29.16	4.62	28.62
66	4 × 16	A × D	10.40	25.54	8.74	27.84	6.81	31.99	6.65	27.39	8.15	28.61
11	3 × 6	A × B	5.82	23.88	4.65	32.53	2.36	28.51	1.77	-	3.23	28.28
65	3 × 20	A × D	10.20	24.07	10.93	27.60	6.70	31.95	4.80	27.87	8.29	28.16
13	3 × 8	A × B	4.33	24.41	3.56	28.76	1.20	31.74	1.90	25.27	2.33	27.79
69	4 × 19	A × D	10.00	26.33	10.98	30.56	7.77	26.87	4.74	26.01	8.49	27.78
125	10 × 20	B × D	7.73	24.30	6.63	27.95	4.49	28.75	3.53	28.36	5.38	27.76
7	2 × 7	A × B	5.12	26.40	5.80	28.62	4.12	25.93	3.34	27.78	4.38	27.71
Trial Mean			8.75	22.47	8.57	26.51	6.10	25.52	4.87	24.30	7.07	24.70
Mean top 15			8.04	25.82	7.94	30.04	5.27	30.35	4.19	27.59	6.29	28.88
Min			3.84	17.83	3.56	20.43	1.08	19.04	1.37	19.45	2.33	18.93
Max			12.59	28.32	11.24	32.77	8.93	35.38	7.31	32.58	9.40	31.45
LSD$_{0.05}$			1.40	2.68	1.61	2.90	1.12	2.97	1.43	3.27	1.00	0.87
Heritability			0.87	0.75	0.83	0.82	0.91	0.83	0.76	0.73	0.91	0.85
σ^2 G			3.76	7.33	3.64	11.09	3.33	12.91	2.15	8.81	2.79	7.59
σ^2 G × E			-	-	-	-	-	-	-	-	0.49	2.48
Residual			1.08	4.84	1.47	4.97	0.66	5.29	1.39	6.68	1.11	1.07

σ^2 G, Genotype variance and σ^2 G × E, the interaction between genotype and environment was significant at α = 0.001. GY and Zn = Grain yield and Kernel zinc concentration, respectively. The least significant differences (LSDs) are for comparing the means among hybrids. Group A, B and D = high Zn QPM line, low Zn non-QPM line, and high Zn non-QPM line, respectively.

The Pearson correlation coefficient values between pairs of traits ranged from −0.14 for Zn and plant height to 0.20 between Zn and days to anthesis (Table S3). Correlation values between Zn and flowering dates (anthesis and silking date) were low (0.16–0.20) but significantly different from zero at *p*-value <0.05 in each environment. Across environments, there was no significant correlation between Zn and any other trait.

The lack of an association between Zn and other traits is promising for maize breeding. Across environments, 15 hybrids were ranked in the top 10% for Zn (Table 2). Those hybrids involved at least one inbred from the high-Zn group (QPM or non-QPM), had 12%–27% Zn content above mean of all hybrids in all environments (24.70 µg/g), and were produced from 13 inbred parents. Five inbreds were from the high-Zn QPM group and four inbred lines each were from the high-Zn non-QPM and low-Zn QPM groups. Six of the 15 hybrids were exclusively produced from QPM inbred lines, while nine were from crosses between QPM and non-QPM inbred lines. Inbred 2 from the high-Zn QPM group and inbred 20 from the high-Zn non-QPM group were parents to four hybrids each.

Among the top 10% of hybrids with high-Zn across environments, high-yielding hybrids with 7.80–9.40 t ha^{-1} of grain were identified (Table 2). Inbred lines 1, 2, 3, and 4 from the high-Zn QPM group and 16, 17, 19, and 20 from the high-Zn non-QPM group were parents to those hybrids. However, despite the lack of correlation between grain yield and Zn, some of the hybrids that showed high-Zn concentration across environments were low-yielding. Overall, grain yield averages of the top 10% hybrids for Zn were 8%–13% lower compared to the averages for all hybrids in each environment and across environments.

The average values of Zn for each inbred line as measured in their hybrids were estimated for each environment and across environments (Table 3). Based on those hybrids, average values for Zn among the four groups of inbred lines (A: high-Zn QPM, B: low-Zn QPM, C: low-Zn non-QPM, and D: high-Zn non-QPM) ranged from 21.15 to 27.97 µg/g. In all environments, the highest average value for Zn corresponded to high-Zn QPM inbreds (26.00 µg/g), while the lowest average value of Zn was recorded for low-Zn non-QPM inbreds (22.96 µg/g).

The genetic potential of the inbreds to serve as parents was assessed exclusively on the basis of their hybrid progenies (Table 3). The top five mean values for Zn in each environment and across environments involved inbred lines 1 and 2. The highest mean value for grain yield was observed for 14 hybrids, which had inbred 13 as one of the parents. Genotypes with higher levels of Zn and grain yield were evident based on the performance of hybrids across the environments. Hybrids 51 and 60 were among the top 10 hybrids with high Zn and grain yield (Table S3). Based on the average grain yield for inbreds as assessed in hybrid combinations, inbreds 1, 2, 16, and 20, which were parents to hybrids 51 and 60, attained grain yields of ≥7 tons ha^{-1} (Table 3).

Analyses of means for Zn and grain yield were conducted for the hybrids across sets (Table 4). Values for average Zn ranged between 19.72 µg/g for low-Zn (QPM × non-QPM) hybrids to 29.72 µg/g for high-Zn (QPM × non-QPM) hybrids. The set of hybrids formed from high-Zn inbreds (QPM × non-QPM) had the highest mean for Zn while the set formed from low-Zn inbreds (QPM × non-QPM) attained the lowest mean for Zn. The average values for grain yield across the sets of hybrids ranged from 3.35 t ha^{-1} for high-Zn QPM × low-Zn non-QPM to 10.57 t ha^{-1} for high-Zn QPM × non-QPM hybrids (Table 4). Similarly, the set of hybrids formed from high-Zn inbreds (QPM × non-QPM) had the highest mean for grain yield, while hybrids produced from QPM inbreds (high-Zn × low-Zn) had the lowest mean for grain yield.

Table 3. Average kernel Zn and grain yield for inbred lines as observed in hybrid progenies.

Inbred Line	Group	Hybrid Zn Levels (µg/g)					Hybrid GY across Environments
		Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Across Environments	t ha^{-1}
1	A	24.31 (15)	27.83 (15)	27.26(15)	25.10 (15)	26.30 (15)	7.16 (15)
2	A	24.43 (15)	29.09 (15)	27.91 (15)	26.01 (15)	27.08 (15)	7.59(15)
3	A	22.58 (15)	27.64 (15)	26.57(15)	24.78 (15)	25.44 (15)	7.32 (15)
4	A	23.15 (15)	27.45 (15)	26.35 (15)	24.59 (15)	25.43 (15)	7.36 (15)
5	A	24.23 (15)	27.83 (15)	25.53 (15)	24.83 (15)	25.77 (15)	7.12(15)
		23.74	**27.97**	**26.72**	**25.06**	**26.00**	**7.31**
6	B	22.90 (15)	28.23 (15)	26.44 (15)	24.48 (15)	25.64 (15)	5.53 (15)
7	B	22.73 (15)	25.78 (15)	25.40 (15)	24.63 (15)	24.67 (15)	5.54 (15)
8	B	23.56 (15)	27.30 (15)	26.16 (15)	25.48 (15)	25.73 (15)	6.61 (15)
9	B	22.21 (15)	25.53 (15)	25.45 (15)	24.79 (15)	24.48 (15)	6.74 (15)
10	B	22.44 (15)	28.41 (15)	25.51 (15)	24.75 (15)	25.27 (15)	6.61 (15)
		22.77	**27.05**	**25.79**	**24.83**	**25.16**	**6.21**
11	C	21.65 (15)	26.23 (15)	25.08 (15)	22.56 (15)	23.75 (15)	7.16 (15)
12	C	20.93 (15)	24.31 (15)	22.51 (15)	23.16 (15)	22.49 (15)	6.63 (15)
13	C	22.9 1(14)	27.25 (14)	25.81 (14)	24.79 (14)	25.21 (14)	7.99 (14)
14	C	20.35 (15)	22.83 (15)	22.97 (15)	22.78 (15)	21.94 (15)	6.63 (15)
15	C	19.94 (14)	23.15 (14)	21.86 (14)	22.00 (14)	21.41 (14)	7.38 (14)
		21.15	**24.75**	**23.64**	**23.06**	**22.96**	**7.16**
16	D	23.93 (14)	27.46 (14)	27.99 (14)	26.99 (14)	26.52 (14)	7.30 (14)
17	D	22.49 (14)	26.63 (14)	25.95 (14)	24.96 (14)	25.02 (14)	6.67 (14)
18	D	21.41 (15)	26.64 (15)	25.41 (15)	24.83 (15)	24.53 (15)	7.68 (15)
19	D	22.67 (15)	26.52 (15)	25.31 (15)	24.23 (15)	24.66 (15)	7.31 (15)
20	D	22.93 (15)	27.23 (15)	28.51 (15)	25.01 (15)	25.98 (15)	7.72 (15)
		22.68	**26.90**	**26.63**	**25.00**	**25.34**	**7.34**
LSD $_{0.05}$		1.19	1.30	1.66	0.94	1.32	0.64

Hybrid Zn levels = average value of kernel Zn as observed in the hybrids that had a given inbred line as a parent. The number of hybrids evaluated for each inbred line is in parentheses.
Hybrid GY (tons ha^{-1}) = average value of GY as observed in the hybrids that had a given inbred line as a parent. The number of hybrids evaluated for each inbred line is in parentheses.
The least significant difference (LSD) is used for comparing the averages (in bold) among groups.

Table 4. Averages for grain yield and Zn concentration for the sets of maize hybrids.

Set Composition	GY (t ha^{-1})					Zn (µg/g)				
	Tlalti 2015	Tlalti 2016	Agua Fria 2015	Cotaxtla 2015	Average across Environments	Tlalti 2015	Tlalti 2016	Agua Fria 2015	Cotaxtla 2015	Average across Environments
Group A × Group B	7.24	6.79	4.35	3.35	5.42	24.49	29.04	27.33	26.71	26.93
Group A × Group C	9.40	9.70	7.63	5.66	8.09	22.82	26.21	23.90	22.09	23.74
Group A × Group D	10.57	10.29	7.39	5.68	8.49	25.14	29.69	29.72	27.31	27.96
Group B × Group C	8.04	8.43	6.04	4.90	6.85	19.72	23.48	21.72	21.28	21.55
Group B × Group D	8.76	8.27	5.38	4.70	6.79	22.69	27.14	26.86	25.42	25.56
Group C × Group D	8.35	8.15	5.92	4.88	6.83	20.21	23.84	23.69	22.93	22.70
LSD $_{0.05}$	0.55	0.54	0.47	0.49	0.14	0.79	0.90	0.94	0.90	0.32

Values for grain yield (GY) and kernel Zn concentration (Zn) were significant at $\alpha = 0.001$ and 0.01. Tlalti = Tlaltizapan. Group A, B, C, and D = high-Zn QPM, low-Zn QPM, low-Zn non-QPM, and high-Zn non-QPM, respectively. Each set consisted of 25 hybrids made by crossing each of five lines from one group to all five lines in the other group. The least significant differences (LSDs) are for comparing the averages among sets.

Variances of general combining ability (GCA) effects, i.e., female, male, or both, and specific combining ability (SCA) effects, i.e., female × male, differed among the six sets of hybrids for all traits (Table 5). For Zn, significant variances due to GCA (female, male, or both) were observed in five of the six sets. The SCA effects were significant only in set four (low-Zn QPM × non-QPM). Partitioning the variances in each set and across the four environments, GCA (GCA$_m$ plus GCA$_f$) accounted for 76% to 96% of the variation observed in Zn (Table S5).

Table 5. Analysis of variance of general combining ability (GCA) and specific combining ability (SCA) effects for grain yield and Zn concentration.

Source of Variation	Set1	Set2	Set3	Set4	Set5	Set6
	Group A × Group B	Group A × Group C	Group A × Group D	Group B × Group C	Group B × Group D	Group C × Group D
			Grain yield			
GCA$_f$	ns	ns	ns	**	***	***
GCA$_m$	***	ns	ns	*	ns	ns
SCA	***	ns	ns	***	***	*
			Zn concentration			
GCA$_f$	ns	***	*	ns	ns	***
GCA$_m$	*	**	*	***	ns	***
SCA	ns	ns	ns	*	ns	ns

*, **, and *** significant at *p* < 0.05, 0.01, and 0.001, respectively; ns = not significant (*p* > 0.05). GCAf = the general combining ability effect of the lines designated as females; GCAm = the general combining ability effect of the lines designated as males. Group A, B, C, and D = high-Zn QPM, low-Zn QPM, low-Zn non-QPM, and high-Zn non-QPM, respectively.

Six inbred lines showed positive GCA effects for Zn (Table 6). Among the six, two inbred lines, 2 and 8, were QPM, and four, 11, 13, 16, and 20, were non-QPM. Inbred lines 1, 2, 16, and 20 were parents to hybrids that attained ≥30 µg/g of Zn across environments (Table S3). The significant and positive GCA effects indicated the inbreds would contribute favorable alleles for Zn in a breeding program if used as males or females, irrespectively. Of the two QPM inbred lines, line 2 was a member of the high zinc group. Of the four non-QPM lines, 16 and 20 were from the high zinc group. Inbred line 16 showed positive GCA for Zn and zero or negative GCA for grain yield (Table 6). Forty-one hybrids had positive SCA for kernel Zn (Table S7).

Table 6. General combining ability (GCA) effects for grain yield and Zn across environments.

Inbred Line	Grain Yield			Zn Concentration		
High Zn, QPM	LZn, QPM	LZn, Non-QPM	HZn, Non-QPM	LZn, QPM	LZn, Non-QPM	HZn, Non-QPM
1	0.00	−0.04	0.00	0.03	0.00	0.26
2	0.00	0.14	0.00	0.01	1.63 **	1.18 *
3	0.00	−0.03	0.00	0.02	−1.40 *	−0.51
4	0.00	0.06	0.00	−0.05	0.03	−0.51
5	0.00	−0.13	0.00	0.00	−0.26	−0.42
Low Zn, QPM	HZn, QPM	LZn, non-QPM	HZn, non-QPM	HZn, QPM	LZn, non-QPM	HZn, non-QPM
6	−2.28 *	0.23	1.16	0.51	0.00	0.02
7	−1.44	−0.14	0.33	−0.16	−0.63	−0.09
8	−0.51	0.67	1.28	0.28	0.48	0.13
9	1.80	−0.46	−0.45	−0.50	−0.10	−0.18
10	2.43 *	−0.30	−2.31 *	−0.13	0.25	0.12
Low Zn, non-QPM	HZn, QPM	LZn, QPM	HZn, non-QPM	HZn, QPM	LZn, QPM	HZn, non-QPM
11	0.00	0.00	0.26	0.44	0.68	1.01
12	0.00	0.00	−1.04 *	−0.38	−0.27	−0.73
13	0.00	0.00	1.02 *	2.34 **	2.53 **	2.53 **
14	0.00	0.00	−0.71	−1.00	−1.16	−1.16
15	0.00	0.00	0.47	−1.40	−1.71 *	−1.71
High Zn, non-QPM	HZn, QPM	LZn, QPM	HZn, non-QPM	HZn, QPM	LZn, QPM	LZn, non-QPM
16	−0.06	0.00	−0.08	1.55	0.34	1.43 *
17	−0.50	0.00	−0.31	−0.63	−0.84	0.40
18	0.20	0.00	0.28	−1.01	0.13	−1.33 *
19	0.05	0.00	−0.11	−0.59	−0.26	−0.95
20	0.32	0.00	0.23	0.73	0.63	0.45

* and ** significant at $p < 0.05$ and 0.01. Lines from one group were mated in a modified mating scheme to lines from three other groups so that three independent estimates of combining ability were computed for each line. LZn, QPM and LZn, non-QPM = low zinc QPM and non-QPM lines, respectively; HZn, QPM and HZn, non-QPM = high Zn QPM and non QPM lines, respectively.

4. Discussion

The inbreds' phenotype may provide useful information for creating hybrids with elevated levels of Zn in the kernel. In this study, hybrids with a Zn content ≥30 µg/g across environments were produced exclusively from inbred lines classified as high-Zn parents, such as inbred 1 and 2 from the high-Zn QPM group and 16 and 20 from the high-Zn non-QPM group. Similar observations were reported in maize [58] and pearl millet [59–62]. However, the Zn levels were lower for all hybrids derived from high-Zn lines compared to the respective values observed in their parental inbred lines. This is consistent with previous studies in maize [58,63] and pearl millet [60,62], which reported significantly lower Zn in hybrids compared to their parental inbred lines. Therefore, an additional criterion, evaluation in hybrid combinations, should be considered when selecting inbred lines for use as parents of hybrids with higher Zn content.

Nutritional improvement in crop plants, including Zn-enriched maize hybrids, may result in a yield penalty [8]. However, previous studies have reported that yield and nutritional traits, such as kernel Zn, could be improved simultaneously [64–66]. In this study, grain yield was not correlated with Zn (r = 0.02). Similar observations were reported in previous studies of maize [27,58,67,68] and pearl millet [61]. Lack of correlation between grain yield and Zn suggested the possibility of improving maize for Zn concentration without reducing the grain yield potential of the hybrids. Consistent with previous studies, hybrids with elevated Zn and grain yield have been reported [7,58,69].

Hybrids developed from mating high-Zn (QPM × non-QPM) inbreds had enhanced levels of Zn concentration. Increased levels of Zn have been reported for QPM germplasm compared to non-QPM germplasm [18,39,40,42]. The dominant, wild-type allele of the *o2* locus codes for a transcriptional factor that regulates the synthesis of zeins [70]. In genotypes homozygous recessive at the *o2* locus, there is a decrease in α-zein [71] with a proportional increase of non-zeins such as albumins, glutelins, and globulins [72]. Those non-zeins are known to bind Zn in the endosperm [73]. Thus, in QPM inbreds, and possibly in some of their hybrids, the elevated levels of Zn could be attributed to reduced levels of zeins and relatively higher levels of other Zn-binding proteins [40].

Higher levels of Zn have also been reported in non-QPM inbred lines [43,47–49,74]. For such inbreds, and perhaps in their hybrids, higher Zn levels could be attributed to genetic factors unrelated to the *o2* locus. Therefore, it might be helpful to explore other mechanisms that can potentially account for high-Zn in those genotypes. During grain filling, metal-binding proteins such as metallothioneins, phytochelatins, and nicotianamine are thought to bind Zn in large amounts [75]. The storage capacity of those binding proteins could possibly be associated with the amount of Zn that accumulates in a maize kernel. Genotypes with a high capacity for Zn storage may possess more Zn-binding proteins. Consequently, enhanced levels of Zn may be achieved in genotypes with more Zn-binding proteins than genotypes with fewer Zn-binding proteins [40]. Instead, enhanced levels of Zn in those hybrids may be attributed to the increase in Zn-binding capacity because of the metal-binding proteins.

In addition, other possible sources of higher levels of Zn in kernels may be attributable to disproportionate growth of the endosperm and embryo. In maize, approximately 49% of the total kernel Zn is in the embryo and the remainder is in the endosperm [19]. If either tissue grows in an unexpected and disproportionate manner, the total amount of Zn in the kernel could be affected. Inbred lines and their hybrid progeny often display different phenology and durations of developmental stage. For example, it is well-known that inbred lines flower later and are shorter than their hybrid progeny. Thus, in addition to the possibility of Zn-binding proteins, the relative proportions of embryo and endosperm in the hybrid progeny should be considered in future investigations.

Understanding the nature of gene action responsible for Zn accumulation in maize kernels could be important in designing an effective breeding strategy for hybrids with increased Zn. The GCA effects accounted for ≥70% of the total variability, suggesting that the accumulation of Zn in maize kernels is predominantly governed by additive gene effects. Similar results were reported in maize [26–28,32,47,76], pearl millet [59–61], rice [77,78], sorghum [79], and wheat [80].

With predominance of variance due to GCA, hybrids with enhanced Zn levels can be obtained by crossing parents with positive GCA effects [81,82].

Among the 10 inbred lines that were originally classified as high-Zn parents, only three inbreds, namely, inbred 2 from the high Zn-QPM group, and 16 and 20 from the high-Zn non-QPM group, had positive GCA effects. This observation was contrary to an earlier study involving 14 inbred lines in which positive GCA effects were observed for all high-Zn parents (seven), while significantly negative GCA effects were detected for the low-Zn lines [28]. In addition, positive GCA effects for Zn were detected for inbreds 8, a low-Zn QPM, and 11 and 13, both from the low-Zn non-QPM group. The positive GCA for Zn observed for inbred lines 2, 8, 11, 13, 16, and 20 suggest the possibility of transmitting favorable alleles from these parental lines to their hybrids and could be useful for breeding to improve Zn content.

Kernel Zn is a phenotype determined late in the development of a maize crop, subject to environmental influences, requiring extensive sample preparation, trained analysts, and costly equipment. Therefore, it could be helpful to identify a secondary trait that can potentially be used for indirect selection of Zn. During growth and development of a maize plant, the vegetative parts serves as a primary source of Zn for kernels. Consequently, plant height could conceivably be used as a secondary trait associated with Zn in kernels. More Zn may be remobilized to the kernels of taller plants than kernels of shorter plants. However, in this study, there was no correlation between plant height and Zn concentration as noted in previous research [68].

5. Conclusions

In summary, the general categories of the inbreds' phenotype, groups A–D, may provide some useful information for developing hybrids with increased Zn content, although more reliable and relevant results can be obtained by evaluating the inbreds in hybrid combinations. Hybrids derived from crossing QPM inbred lines alone had greater mean values for Zn (26.93 µg/g) than hybrids derived from crossing non-QPM inbred lines (22.70 µg/g). However, hybrids with the highest mean values for Zn were observed when high-Zn QPM inbred lines were crossed with high-Zn non-QPM inbreds (hybrids 51, 56, and 60 had ≥30 µg/g of Zn). Six inbred lines with positive and/or significant GCA for Zn were identified. These results indicate some potential to develop high-Zn hybrids using a combination of QPM and non-QPM inbred lines. The largest proportion of variability for Zn among hybrids was due to GCA effects, suggesting that additive gene effects were more important than non-additive gene effects for Zn in this set of germplasm.

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