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RESEARCH

Genotype and Nitrogen Effects over Maize Kernel Hardness and Endosperm Zein Profiles

José A. Gerde,* Santiago Tamagno, Juan C. Di Paola, and Lucas Borrás

ABSTRACT

Maize (*Zea mays* L.) kernel hardness is of utmost importance for dry-milling processors. Zeins, maize prolamins, are known to be key proteins affecting this trait. We investigated the response of kernel zein profiles to N fertilization in maize hybrids with contrasting kernel hardness (measured as test weight, vitreousness, kernel density, and floaters percentage). A field experiment was done during two seasons, with three N fertilization treatments and four commercial hybrids with different hardness (two flint and two dent kernel types). We also measured yield, kernel protein concentration, and zein profiles (Z2, or γ and β, and Z1, or α and δ). Flint kernel type always yielded less and showed higher kernel hardness indicators when compared with dents ($P < 0.01$). N fertilization helped increase yield and kernel hardness in both kernel types. Flint kernel type had consistently more Z2 than dents, while only in 1 yr Z1 was higher in flints. Dent kernel type had a Z1/Z2 ratio similar to or higher than flints. Increasing N resulted in increased concentration of both zein types, but the effect was more pronounced on Z1. Significant correlations were observed between the different zein types (Z1 and Z2) and hardness indicators and total protein concentration, but Z2 showed the highest correlations with all kernel hardness traits. Our results expand previous knowledge on genotype and N fertilization effects over zein profiles and their involvement in kernel physical characteristics relevant for dry milling.

J.A. Gerde, S. Tamagno, and L. Borrás, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Campo Experimental Villarino S/N, Zavalla (S2125ZAA), Prov. de Santa Fe, Argentina; and J.C. Di Paola, Cotecna Inspección Argentina S.A., Av. Alicia Moreau de Justo 170, piso 2, Ciudad Autónoma de Buenos Aires (C1107AAD), Argentina. Received 27 Aug. 2015. Accepted 23 Nov. 2015. *Corresponding author (jose.gerde@unr.edu.ar).

Abbreviations: GMO, genetically modified organism; MAP, monoammonium phosphate; SS, sum of squares.

Maize endosperm hardness is an important quality attribute for several grain processors, especially for the dry milling industry. Harder kernels result in higher milling yields of large flaking grits, one of the main targets of this process (Litchfield and Shove, 1990). Kernel hardness also impacts directly on several kernel nutritive properties, on grinding power requirements, and dust formation (Paulsen et al., 2003). High test weight, high kernel density, low floaters percentage, and high vitreous/floury endosperm ratio are all attributes typical of hard endosperm maize, and are related to high dry milling yields (Kirleis and Stroshine, 1990; Wu and Bergquist, 1991; Cirilo et al., 2011; Blandino et al., 2013).

Genotype differences in kernel hardness are common, and several industries are willing to pay premiums for these differences. Argentina, for example, has developed a supply chain of non-GMO (genetically modified organism) hard endosperm flint maize, also known as plata maize, which results in a yearly average of 350 thousand metric tons of flint maize exported to the European Union during the last decade. The physicochemical characteristics of these flint maize kernels make them a preferred raw material for the dry milling industry (Rooney and Serna Saldívar, 2003), and the European Union has issued some special import permits for flint maize from Argentina, the United States, and Canada (European Commission, 1997). However, the introduction

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of dent germplasm has contributed to large increases in farmers' grain yield with concomitant decreases in grain hardness. As in many countries, Argentinean maize has slowly moved to softer, more yellow kernels (Brun and Dudley, 1989). The production of hard endosperm flint grain is currently produced through special contract growing agreements between farmers and industry. In this context, there is a general need for understanding what is controlling kernel hardness, and its relationship with yield.

Reaching optimum grain hardness standards is feasible, but needs to be coupled with high physical yields on farmer fields. Farmers are paid premiums for producing special hard endosperm maize at many production systems (U.S. Grains Council, 2006). In Argentina, for example, current regular dent hybrids are out-yielding flint kernel types across a wide range of growing conditions (Tamagno et al., 2015), and premiums cover this yield gap. Breeders selecting for hard endosperm maize and large yields on farmer fields have the limitation of maintaining minimum quality standards. In addition to the strong genetic control of kernel hardness, the environment also affects this trait (Watson, 2003). To avoid situations where specific fields cannot reach minimum quality standards demanded by the dry milling supply chain, production systems combine genotype selection with specific crop management.

Kernel hardness has traditionally been correlated with specific endosperm proteins, the prolamins, also commonly called zeins (Robutti et al., 1997; Pratt et al., 1995; Fox and Manley, 2009). Wilson (1991) defines zeins as alcohol-soluble proteins of maize kernel endosperm that may or may not require reduction before extraction. Zein accumulates in endosperm protein bodies that concentrate on the peripheral zone of the endosperm, decreasing toward the flourier endosperm center (Wolf et al., 1969). Zeins were classified according to their solubility as α-, β-, γ- and δ-zein (Esen, 1986). Lending and Larkins (1989) proposed a model in which α- and δ-zeins are located in the interior part of the protein bodies, and γ- and β-zeins accumulate on the protein body surface. Zein-2 (Z2), the compendium of γ- and β-zein, has been associated with horny endosperm fractions (Eyhérabide et al., 1996; Robutti, 1995; Robutti et al., 1997). In particular, the major component of Z2, 27 kDa γ-zein (peak 2 according to Robutti et al. (1997), or zein E according to Wilson, 1991) has been specifically related to hardness attributes (Eyhérabide et al., 1996). Another subgroup, zein-1 (Z1, α- and δ-zeins), was also found in higher proportion in horny endosperms when compared with softer ones at other studies (Dombrink-Kurtzman and Bietz, 1993).

Maize zein concentration within kernels is known to depend mostly on the genotype (Motto et al., 1996; Flint-Garcia et al., 2009), but zein concentration can vary as a result of the environmental conditions that crops experience in the field. Nitrogen fertilization is a common management practice in maize known to affect kernel protein concentration (Tsai et al., 1978; Pearson and Jacobs, 1987; Uhart and Andrade, 1995). In general, any crop growing condition that produces more assimilate availability per kernel during the filling period increases kernel protein concentrations (Borrás et al., 2002). Protein increases have usually resulted in higher kernel hardness indicators, like higher test weights and lower floaters percentage (Cirilo et al., 2011). These last two parameters are widely used by the maize dry milling industry to grade their raw materials. Nitrogen fertilization is also known to affect the concentration of zein in the maize endosperm under field (Tsai et al., 1978, 1984; Ahmadi et al., 1995) and in vitro (Singletary et al., 1990) growing conditions. The relative response of the different zein fractions to N fertilization levels, however, is not known.

We are particularly interested in establishing links between kernel hardness and specific zein changes, and understanding how specific crop management factors affect their relationships. There are premiums paid for hard endosperm maize by industries at many production systems, and it is evident that to manipulate maize hardness there is a need to dissect the relationships between zein profiles and kernel hardness. Our objectives were (i) to assess the effect of N fertilization on the zein profile of maize genotypes known to differ in their kernel hardness, and (ii) to understand the relationships between endosperm zein profiles and physicochemical industrial quality attributes of the kernel for dry milling.

MATERIALS AND METHODS

Field Experiments

A field experiment was conducted at the experimental field of Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, in Zavalla, Santa Fe, Argentina (33° 1′ S, 60° 53′ W). Four commercial genotypes and three levels of N application were combined in a complete randomized block design with three replicates. The experiment was conducted during two growing seasons (2012/2013 and 2013/2014). Genotypes included two regular GMO dents (DK747 and AX887) and two non-GMO hard endosperm flints (ACA2002 and NT426). Genotypes represent widely planted genotypes for both kernel types at the central Argentinean region. Planting dates were 21 Sept. 2012 and 2 Oct. 2013.

Individual plot replicates were four rows with 0.52 m row spacing and 6 m long. Only the two central rows were harvested and used for sampling. A uniform stand density of 8 plants m^{-2} was used across treatments, and plots were overplanted and hand-thinned at V2 to V3. Plots were managed following common agronomic practices for the region for weeds and diseases, and grown under rainfed conditions. Rainfall from planting date to physiological maturity was different across years. Both years showed similar rainfall distribution, but the total rainfall amount was quite different (681 and 391 mm for Years 1 and 2, respectively). Average temperatures were also different (21.1 and 23.0°C for Years 1 and 2, respectively), making year 2 a warmer and dryer growing season. Because genotypes were different for insect resistance, we carefully controlled any insect presence with recommended products throughout growing seasons.

Soil samples (0 to 60 cm) were taken before planting and analyzed for N-NO_3. At planting, monoammonium phosphate (MAP, 10–50–0, N–P–K) was applied at a rate of 120 kg ha^{-1} to all plots. Subsequently, three N treatments were established: (i) low, with no N added other than MAP; (ii) intermediate, with urea added to adjust soil N level to 155 kg N ha^{-1} (N from the soil plus MAP plus urea); and (iii) high, with urea added to adjust the soil N level to 250 kg N ha^{-1} (N from the soil plus MAP plus urea). At the low-level treatment, the soil reached 85 kg N ha^{-1} across years (N from the soil plus MAP). The urea was broadcasted manually over the plots soon after thinning (V3 to V4).

At commercial maturity, the two central rows per plot were manually harvested and mechanically threshed. Yield was calculated and presented on a 14.5% moisture basis.

Kernel Analyses

A series of kernel analyses was done over each individual plot replicate. Test weight was measured after kernel sample homogenization, with a cone sample divider using a 250 mL Schopper chondrometer (Cuenca, Rosario, Argentina). Results were expressed as kg hL^{-1}.

Floaters percentage (%) was measured using an aliquot of 100 sound selected kernels submerged in a $NaNO_3$ solution (density: 1.25 g cm^{-3}) at 35°C and thoroughly shaken every 30 s for 5 min to eliminate any bubbles. The percentage was calculated counting the number of floating kernels in relation to the total number of kernels used. The test was done five times per field replicate.

Percentage vitreous kernels (%) was determined by dissecting longitudinally 200 sound kernels and visually inspecting them for the absence of dented apical kernel area. There is also the need for central floury endosperm section to be completely surrounded by horny endosperm, and horny endosperm needs to represent a minimum of 50% of the endosperm fraction. The number of kernels complying with these three conditions was divided by the total number of kernels and expressed as percentage vitreousness. Test weight, floaters percentage, and vitreousness were determined according to the methods approved by the European Commission for flint maize imports (European Commission, 1997).

Kernel protein concentration (g 100 g^{-1}) was determined by near-infrared spectroscopy with an Infratec 1241 instrument (Foss, Hillerød, Denmark). Results were expressed on a dry weight basis. For kernel density determination, 20 intact, nondamaged maize kernels were transferred into a 50 mL burette containing 20 mL ethanol. After carefully removing air bubbles, the difference in volume was recorded (Gambín and Borrás, 2005). Kernels were dried in a forced air oven at 65°C for 96 h, and kernel density (g cm^{-3}) was calculated as the ratio between dry weight (g) and volume (cm^3).

Reduced zein profiles were determined as described by Borrás et al. (2006) with modifications, after grinding 100 maize kernels with a laboratory grinder (Tecnodalvo, Buenos Aires, Argentina). A 200 mg flour aliquot was defatted twice with 1 mL hexane under constant agitation (140 rpm) for 1 h. After each extraction, the milled maize was centrifuged at 12,000 *g* and 5°C for 10 min. The supernatant was discarded and the remaining pellet was left overnight for residual hexane evaporation. The defatted material was extracted with 1 mL of 70% ethanol containing 5% β-mercaptoethanol and 0.5% sodium acetate for 2 h at room temperature and 140 rpm. The extract was centrifuged at 12,000 *g* and 5°C for 10 min, and the supernatant was diluted 1:5 with the extraction solvent and filtered (0.22 μm pore nylon syringe filter). The filtrate was analyzed by reversed phase high-performance liquid chromatography using a Dionex Ultimate 3000 system equipped with a 4.6 × 250 mm 218MS C18 column with a 300 Å pore size (Vydac, Grace Davison Discovery Sciences, Deerfield, IL). The mobile phase system was acetonitrile (Solvent A) and water (Solvent B), both containing 0.10% trifluoroacetic acid. The starting conditions were 28% Solvent A, increasing linearly to 60.5% Solvent A after 50 min and holding at 60.5% Solvent A for another 10 min. The injection volume was 20 μL, the mobile phase flow was established at 1 mL min^{-1}, and detection was set at 210 nm (Eyhérabide et al., 1996). Peak identifications were done using maize public inbred lines B57, N28, A619, and W64A acquired at the USDA Germplasm Collection, and using peak assignment criteria from Wilson (1991) and Eyhérabide et al. (1996; Fig. 1). Peak quantification was performed using peak area relative to the mass (dry basis) of extracted maize (Dombrink-Kurtzman and Bietz, 1993). Flour moisture was determined by weight difference using a 2-g aliquot dried for 2 h at 130°C.

Statistical Analysis

Results for all measured variables were analyzed by ANOVA using PROC GLM from SAS (SAS Institute, 1999). The model included kernel type (flint or dent), genotypes nested within kernel type, N treatment, year, and all possible interactions. They were all considered as fixed effects. Significance level was established at $\alpha = 0.05$, except when mentioned. Percentage sum squares (% sum of squares, SS) were calculated to estimate the contribution of particular effects to total variation. Pearson correlation coefficients between Z1, Z2, and Z1/Z2, and kernel physicochemical parameters were determined using PROC CORR from SAS (SAS Institute, 1999).

RESULTS

Crop Yields

Crop yields showed significant ($P < 0.01$) year, kernel type (flint or dent), N fertilization, and genotype-within-kernel-type main effects. Significant interactions ($P < 0.01$) were year × N fertilization and year × genotype within kernel type (Table 1). Year was the effect that accounted for most yield variations (66% of SS; Table 2).

Kernel type also explained a significant portion of yield variations (16% SS), where dent kernel type always outyielded flint kernel type. Genotypes within each kernel type were significantly different, and this was especially evident when the two flint kernel types were compared. On average, ACA2002 yielded less than NT426 (Table 2).

Positive N fertilization effects were also quite evident for yield. However, this effect was highly dependent on the year ($P < 0.01$). During the first year, increasing N availability resulted in large yield increases, while during the second year no differences were observed (Table 2).

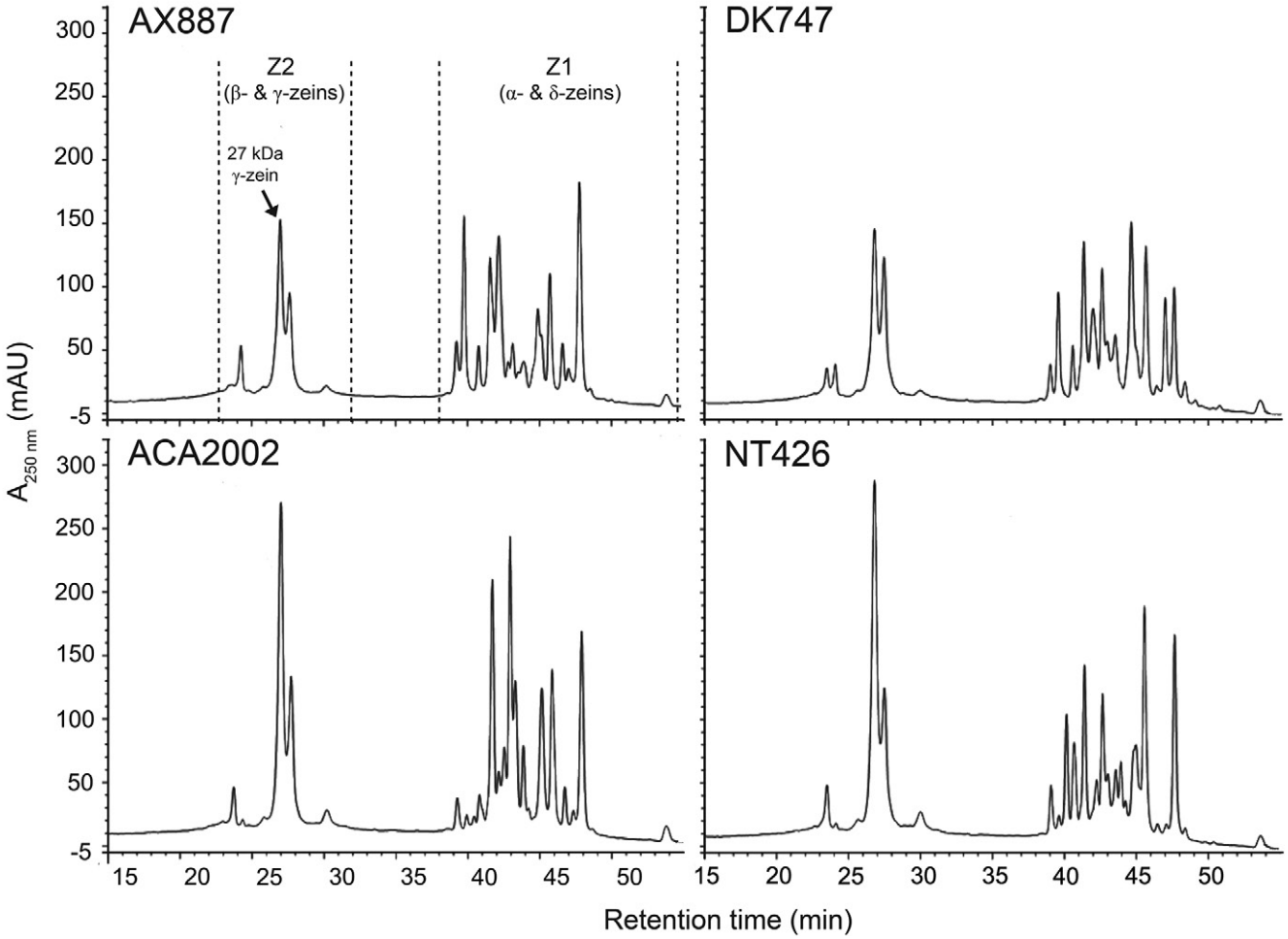

Fig. 1. Chromatograms of zein profiles from four genotypes (dent kernel type AX887 and DK747, and flint kernel type ACA2002 and NT426) grown under a similar environmental condition (Year 1 under high N fertilization). The *y*-axis represents the absorbance at λ = 210 nm expressed in milliabsorbance units (mAU), and the *x*-axis represents the retention time expressed in minutes. Zein-2 (Z2), which includes β- and γ-zeins, and zein-1 (Z1), which includes α- and δ-zeins according to Wilson (1991), are described in the chromatogram corresponding to AX887.

Table 1. Effect of experimental year, kernel type, genotype, N fertilization, and their interactions on yield, kernel hardness attributes, and specific zein concentration.

Source of variation	Yield	Test weight	Floaters	Vitreousness	Kernel density	Protein	Z1	Z2	27 kDa γ-zein	Z1/Z2 ratio
	kg ha^{-1}	kg hL^{-1}	%		g cm^{-3}	g 100 g^{-1}	area units g^{-1}			
Year (Y)	***	***	*** (5)	***	***	***	***	***	***	***
Kernel type (KT)	*** (458)†	***	***	***	***	***	***	***	***	***
Genotype (Kernel type)	***	***	** (7)	***	*	***	***	***	***	***
Nitrogen (N)	***	*	***	***	*	*** (0.2)	***	***	***	***
Y × KT	–	** (0.43)	–	***	–	** (0.3)	***	***	***	***
Y × Genotype (Kernel type)	* (917)	–	–	*** (7)	* (0.026)	*** (0.4)	* (177)	***	**	*** (0.2)
KT × N	–	–	** (8)	–	–	–	–	–	–	–
Y × N	*** (793)	–	–	–	–	–	**	**	***	***
N × Genotype (Kernel type)	–	** (0.75)	–	–	–	–	–	–	***	–
Y × KT × N	–	–	–	*** (8)	* (0.032)	–	*** (217)	** (72)	*** (30)	–
Y × N × Genotype (Kernel type)	–	–	–	–	–	–	–	*** (102)	*** (43)	* (0.3)

* Significant at $P < 0.05$.

** Significant at $P < 0.01$.

*** Significant at $P < 0.001$.

† Numbers in parentheses represent the least significant differences (LSD) of the means presented in Tables 2, 3, and 4.

Table 2. Yield for flint (ACA2002, NT426) and dent (AX887, DK747) kernel type genotypes grown at three N fertilization levels during 2 yr. Statistical treatment effects are described in Table 1.

Year	Kernel type	Genotype	N treatment	Yield
				kg ha^{-1}
Year 1	dent	AX887	low	10,040
			intermediate	11,838
			high	13,824
		DK747	low	9,614
			intermediate	12,556
			high	12,969
	flint	ACA2002	low	6,326
			intermediate	9,021
			high	9,501
		NT426	low	7,919
			intermediate	9,705
			high	10,780
Year 2	dent	AX887	low	6,392
			intermediate	5,730
			high	5,979
		DK747	low	5,889
			intermediate	6,850
			high	6,741
	flint	ACA2002	low	3,363
			intermediate	2,137
			high	2,785
		NT426	low	5,528
			intermediate	6,032
			high	5,506
Year 1				10,362
Year 2				5,244
	dent			8,951
	flint			6,463
		AX887		8,967
		DK747		8,935
		ACA2002		5,487
		NT426		7,439
			low	6,902
			intermediate	7,984
			high	8,286

Kernel Hardness

For describing changes in kernel hardness, we measured test weight, floaters, vitreousness, and kernel density (Tables 1 and 3). Test weight was different between kernel types, where flints showed higher values when compared with dents, and the difference between kernel types was slightly larger during the second year, as denoted by a significant year × kernel type significant interaction ($P < 0.01$). Nitrogen fertilization increased test weight values for both flints and dents. Genotypes within each kernel type showed significant test weight differences, and not all genotypes within each kernel type responded similarly to N increases (N × genotype within kernel type significant interaction, $P < 0.01$; Tables 1 and 3).

Kernel type accumulated most of the variation for floaters (43% SS), where flints showed consistently lower values than dents. Nitrogen fertilization consistently decreased floaters, although this effect was larger in flints (N × kernel type significant interaction, $P < 0.01$). And there were genotype differences within each kernel type ($P < 0.01$), where DK747 had more floaters than AX887 among dents, and ACA2002 more floaters than NT426 within flints (Tables 1 and 3).

Vitreousness was also mostly explained by kernel type differences (89% SS), where flints showed consistently higher values than dents. Nitrogen fertilization increased kernel vitreousness, and a triple significant year × N × kernel type interaction showed that flints had a larger response in vitreousness because of N fertilization during Year 1, while dents showed a larger vitreousness increase because of N fertilization during year 2 (Tables 1 and 3).

Kernel density also was highly related to kernel type (31% SS explained by kernel type), where flints had higher values than dents. Nitrogen fertilization increased kernel density across all genotypes, and genotype differences in kernel density within each kernel type were also evident (Tables 1 and 3).

In summary, flint kernel type showed significantly higher kernel hardness (higher test weight, more vitreousness, lower floaters, and higher kernel density), and genotype differences within each kernel type were evident for most traits. Nitrogen fertilization increased kernel hardness both for flint and dent kernel types.

Kernel Protein

Significant differences in protein concentration were observed when flint and dent kernel types were compared. Flints had significantly more kernel protein concentration than dents (approximately 1 g 100 g^{-1} more than dents), and there were clear genotype differences within each kernel type for protein concentration ($P < 0.001$, Tables 1 and 3).

Increasing N fertilization resulted in consistently higher kernel protein concentrations during both growing seasons (Table 3). Kernel protein concentration values during Year 2 were significantly higher than Year 1, and flints increased their values more than dents (significant year × kernel type interaction).

Endosperm Zein Profiles

Different zeins were identified and grouped under Z2 (γ and β) and Z1 (α and δ). For a general comparison, Fig. 1 describes the zein chromatograms of the evaluated four genotypes (dent kernel type AX887 and DK747, and flint kernel type ACA2002 and NT426) grown under a similar environmental condition.

Results showed that Z1 was significantly affected by year, kernel type, genotype within each kernel type, and N fertilization ($P < 0.001$, Table 1). And the interactions

Table 3. Description of kernels physicochemical characteristics of two flint (ACA2002, NT426) and dent (AX887, DK747) kernel type genotypes grown at three N fertilization levels for 2 yr. Statistical treatment effects are described in Table 1.

Year	Kernel type	Genotype	N treatment	Test wt.	Floaters	Vitreousness	Kernel density	Protein
				kg hL^{-1}	%		g cm^{-3}	g 100 g^{-1}
Year 1	dent	AX887	low	76.9	57	5	1.130	7.3
			intermediate	77.9	24	5	1.150	8.6
			high	78.5	18	7	1.154	9.0
		DK747	low	76.8	49	6	1.134	7.9
			intermediate	77.2	38	12	1.156	8.8
			high	76.7	40	6	1.140	9.7
	flint	ACA2002	low	77.2	32	74	1.173	8.6
			intermediate	78.6	16	92	1.136	9.6
			high	78.5	17	89	1.184	9.8
		NT426	low	79.2	17	87	1.204	8.1
			intermediate	78.8	14	94	1.167	8.8
			high	79.3	12	96	1.251	9.8
Year 2	dent	AX887	low	77.8	38	20	1.186	9.3
			intermediate	78.5	19	50	1.208	10.8
			high	79.0	10	60	1.209	11.5
		DK747	low	78.5	51	10	1.157	9.6
			intermediate	78.6	19	20	1.176	10.8
			high	78.4	31	20	1.186	11.0
	flint	ACA2002	low	77.8	14	92	1.208	11.3
			intermediate	78.7	9	96	1.228	12.8
			high	77.5	11	96	1.237	12.9
		NT426	low	79.6	6	93	1.220	9.9
			intermediate	79.4	5	97	1.226	11.4
			high	79.3	8	96	1.217	11.6
Year 1				77.9	28	48	1.165	8.8
Year 2				78.6	18	62	1.205	11.1
	dent			77.9	33	18	1.166	9.5
	flint			78.7	13	92	1.204	10.4
		AX887		78.1	28	24	1.173	9.4
		DK747		77.7	38	12	1.158	9.6
		ACA2002		78.0	17	90	1.194	10.8
		NT426		79.2	10	94	1.214	9.9
			low	77.9	33	48	1.177	9.0
			intermediate	78.4	18	58	1.181	10.2
			high	78.4	18	59	1.197	10.6

year × genotype within kernel type, year × kernel type, year × N, and year × kernel type × N were also significant ($P < 0.05$, Table 1). However, with all these interactions, it is important to note that the effects that accumulated most variation were N fertilization (27% SS), year (12% SS), kernel type (19% SS), genotype within each kernel type (12% SS), and year × kernel type (14% SS).

Increasing N fertilization increased the levels of Z1 in all genotypes; however, the environmental effect (year) on Z1 was stronger over flint than over dent kernel types (significant year × kernel type interaction, $P < 0.01$). Differences in Z1 across N treatments for flint kernel type were larger during Year 2 than Year 1 (Table 4). It is also relevant to note that significant genotype differences within each kernel type for Z1 were evident (Tables 1 and 4).

Significant main effects (year, kernel type, N fertilization, and genotype within kernel type) and interactions (except for N × genotype within each kernel type and kernel type × N effects) were observed on Z2 (Table 1). For Z2, zein kernel type accounted for most variation (73% SS), while the rest of the significant sources of variation accounted for <7% SS each. This result is very different than the one described for Z1 (kernel type explaining 19 vs. 73% SS for Z1 and Z2, respectively). Flint kernel type always had more Z2 than dents when evaluated at the same N condition within the same year, and significant differences were observed between the two flint genotypes (Tables 1 and 4).

When considering 27 kDa γ-zein, the major component of Z2, results are mostly similar to the ones described for Z2 (Tables 1 and 4). Kernel type also accounted for most variation (73% SS).

The Z1/Z2 ratio was calculated to explore relative differences in the proportion of zein types within kernels. Significant effects for the Z1/Z2 ratio were observed for year, kernel type, genotype within each kernel type, and N fertilization ($P < 0.001$). The interactions year × kernel

Table 4. Description of zein-1 (Z1), zein-2 (Z2), the specific Z2 27 kDa γ-zein, and Z1/Z2 ratio for flint (ACA2002, NT426) and dent (AX887, DK747) kernel type genotypes grown at three N fertilization treatments for 2 yr. Statistical treatment effects are described in Table 1.

Year	Kernel type	Genotype	N treatment	Z1	Z2	27 kDa γ-zein	Z1/Z2
				— area units g^{-1} —			
Year 1	dent	AX887	low	1,384	610	256	2.3
			intermediate	1,352	562	217	2.4
			high	1,935	754	342	2.6
		DK747	low	1,309	491	224	2.7
			intermediate	1,661	638	244	2.6
			high	2,077	728	330	2.9
	flint	ACA2002	low	1,698	849	444	2.0
			intermediate	1,675	846	372	2.0
			high	2,068	1,001	560	2.1
		NT426	low	1,430	930	529	1.5
			intermediate	1,318	841	394	1.6
			high	1,885	1,069	646	1.8
Year 2	dent	AX887	low	1,374	571	165	2.3
			intermediate	1,643	615	186	2.7
			high	1,840	625	200	3.0
		DK747	low	1,636	749	224	2.2
			intermediate	1,540	564	196	2.8
			high	2,003	859	263	2.3
	flint	ACA2002	low	2,147	1,002	446	2.1
			intermediate	2,925	1,111	505	2.6
			high	3,053	996	302	3.1
		NT426	low	1,700	1,133	500	1.5
			intermediate	2,246	1,164	501	1.9
			high	2,419	1202	571	2.0
Year 1				1,649	777	380	2.2
Year 2				2,044	889	338	2.4
	dent			1,646	649	237	2.6
	flint			2,047	1,012	480	2.0
		AX887		1,588	625	228	2.5
		DK747		1,704	671	247	2.6
		ACA2002		2,261	968	438	2.3
		NT426		1,833	1,057	523	1.7
			low	1,585	799	349	2.1
			intermediate	1,795	793	327	2.3
			high	2,160	904	402	2.5

type, year × genotype within each kernel type, year × N, and year × N × genotype within each kernel type were also significant (Table 1). As such, it is evident that the proportion of Z1/Z2 ratio was not constant. On average, flints had a significantly lower Z1/Z2 ratio than dents, and N fertilization increased the Z1/Z2 ratio because of its larger effect over Z1 than Z2 (Table 4).

Correlations among Zein Profiles and Physicochemical Quality Factors

Pearson correlation coefficients were determined between Z1, Z2, and the Z1/Z2 ratio with the physicochemical kernel quality traits measured (Table 5). Significant and positive correlations were detected between Z1 and kernel density, vitreousness, and protein concentration. Z1 was negatively correlated with floaters percentage.

Table 5. Pearson correlation coefficients (*r*) between several kernel hardness and composition traits and zein-1 (Z1), zein-2 (Z2), the specific Z2 27 kDa γ-zein, and Z1/Z2 ratio. Each correlation includes 24 data points (four genotypes, three N fertilization levels, and two experimental years).

Trait	Z1	Z2	27 kDa γ-zein	Z1/Z2 ratio
Test weight	ns†	0.58*	0.50*	−0.52**
Floaters	−0.48*	−0.67***	−0.57**	ns
Vitreousness	0.48*	0.82***	0.77***	−0.50*
Kernel density	0.59*	0.69***	0.52**	ns
Protein	0.83*	0.51*	ns	ns

* Significant at $P < 0.05$.

** Significant at $P < 0.01$.

*** Significant at $P < 0.001$.

† ns, nonsignificant.

Z2 was positively correlated with kernel density, test weight, vitreousness, and protein concentration. Z2 was negatively correlated with floaters percentage. The concentration of the specific Z2 27 kDa γ-zein was also significantly correlated with kernel density, test weight, vitreousness, and floaters percentage.

The Z1/Z2 ratio was negatively correlated with test weight and vitreousness.

These results indicated that Z2 explained a greater proportion of all kernel hardness indicators (test weight, vitreousness, floaters, and kernel density) than Z1 or the ratio between Z1 and Z2. The correlations between the concentration of 27 kDa γ-zein and hardness indicators followed the same tendency as Z2, although the correlation coefficients were slightly smaller.

The higher correlation between kernel protein concentration with Z1 than with Z2 helps to understand that kernel protein concentration changes were more associated to changes in Z1 than Z2. This is also in general agreement with the differential Z1 and Z2 response to N fertilization, where N fertilization increased kernel protein concentration and the Z1/Z2 ratio (Tables 3 and 4).

DISCUSSION

The present manuscript reports on how kernel hardness and yield respond to environmental (year), genetic (kernel type, specific genotypes), and management (N fertilization) effects. A relevant result is that grain hardness was maximized at N fertilization levels that were higher than the ones needed for maximum yield, as shown by yield and kernel hardness response to N fertilization during Year 2. In this environment, N fertilization had no effect over crop yields due to limited rainfall, but did increase kernel hardness and kernel protein concentration, showing the importance that this management option has for increasing grain quality regardless of yield increases.

Changes in kernel hardness indicators (test weight, floaters, vitreousness, and kernel density) were correlated with genotype and environmental changes in kernel zein profiles. Dent genotypes yielded more than flints in all environments, and their relative yield differences were in general agreement with other recent studies (Tamagno et al., 2015). Nitrogen fertilization effects on yield and kernel composition were also expected based on the general maize response to this nutrient (Uhart and Andrade, 1995; Ciampitti and Vyn, 2011). Tsai et al. (1978) and Singletary et al. (1990) reported increases in zein content in the developing kernel when additional N was supplied. We expanded these results by determining that increased N supply through crop N fertilization resulted in differential Z1 and Z2 increases (Table 4).

The physicochemical characteristics of maize kernels are of great importance in terms of quality of feedstock for dry milling processing. Dombrink-Kurtzman and Bietz (1993) observed a greater concentration of Z1 in horny endosperm fractions than in floury ones in several maize genotypes. Pratt et al. (1995) studied possible correlations between each Z2 main peak and the compendium of peaks comprising Z1 with kernel density, finding that the correlations between hardness and kernel density and different zein components were very genotype specific. However, Z2 was not studied as a whole. Robutti et al. (1997) proposed that the specific Z2 zein 27 kDa γ-zein (22 kDa E zein, according to Wilson, 1991) and Z1 are the main ones responsible of hardness of maize kernels. Landry et al. (2004) found that the relative abundance of the 27 kDa γ-zein in floury or horny endosperm was genotype dependent. Wu et al. (2010) demonstrated that γ-zeins, and their interaction with starch granules, play a mechanistic role on the determination of the endosperm physical characteristics in quality protein maize. In our study, zein concentrations were studied in whole endosperms, and although the 27 kDa γ-zein contributed to explain kernel hardness attributes, Z2 (the sum of β- and γ-zeins) captured most kernel hardness variability (Table 5). Our results also show that genotype differences in kernel hardness are more related to the Z2 fraction as a whole. It is evident that specific Z2 zeins, like 27 kDa γ-zein, are involved in kernel hardness, but other components of Z2 seem also relevant. This might also explain why transgenic approaches reducing the specific accumulation of 27 kDa γ-zein had negligible effects over test weight and kernel density (Jung et al., 2010).

Lending and Larkins (1989) proposed that Z1 deposition occurred in the inner part of a protein body, which at early grain filling is composed mostly by Z2. Later, during grain filling, protein bodies are filled by Z1, with Z2 accumulating in the peripheral zone. Both Z2 and Z1 could be responsible for changes in hardness; Z2 because of the interactions among protein bodies and between protein bodies and starch granules, and Z1 filling the protein bodies and creating more contact chances. Our results confirm the involvement of both Z2 and Z1 in determining our kernel hardness indicators. When considering a measure of bulk density such as test weight, where not only kernel density but also kernel shape and size are relevant, positive associations only with Z2 were evident (Table 5). The lack of association between Z1 and test weight suggests that this negative correlation is the result of increasing Z2 concentration with little or no influence of Z1.

In the present study, a limited number of genotypes from dent and flint kernel types were studied, grown at three N fertilization conditions during two growing seasons. Because of this limited genotype number, the differences between flint and dent types could be due to genetic factors other than kernel type. We know that these genotypes differ in many canopy growth traits (Tamagno et al., 2015). There is a need for testing a larger genotype diversity, exploring a wider range of kernel hardness attributes. This would provide information to understand the role of the different polypeptides that compose Z1 and Z2 fractions on the development of the kernel physicochemical hardness attributes. But, most important, it is evident that the interaction of the different zeins and kernel starch granules needs to be studied for a better understanding of the development of physicochemical quality attributes of the kernel endosperm.

CONCLUSIONS

Flint kernel type showed consistently lower yield levels, more kernel protein concentration, and higher kernel hardness than dents. This higher kernel hardness was mostly related to a greater proportion of Z2 when compared with dent genotypes.

Increasing N fertilization levels not only augmented grain yield but also improved kernel hardness indicators, especially in hard endosperm flint genotypes. Both Z1 and Z2 increased with N fertilization, and these increases were linearly correlated to kernel protein concentration. However, Z1 was more affected by factors such as year and N fertilization than Z2.

Significant correlations were observed between the different zein types (Z1 and Z2) and hardness indicators, where Z2 showed the highest correlations with kernel hardness. Our results demonstrate how genotype selection and N fertilization management affect zein profiles, and their involvement in kernel physical characteristics relevant for dry milling processing.

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