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32	Abstract	Maize (*Zea mays* L.) grain is an important feedstock for the ethanol-producing industry. However, little is known about the optimum grain quality for optimizing ethanol yielding efficiencies. We specifically investigated the response of ethanol yields (L Mg^{-1}) to kernel hardness, and its physiological determinant endosperm zein protein profiles, as affected by genotype selection, field nitrogen (N) fertilization, and crop growth environment. We measured ethanol yield and related this to different kernel hardness indicators, kernel composition, and zein profiles. We also described changes in field ethanol yield (L ha^{-1}), by taking into account the crop yield (Mg ha^{-1}). Hard endosperm genotypes always yielded less ethanol than softer endosperm ones per grain mass (L Mg^{-1}). Higher N fertilization rates increased kernel hardness and decreased ethanol yield (L Mg^{-1}) on soft endosperm dented genotypes but had no effect on hard endosperm ones. Ethanol yield was negatively correlated with kernel density, kernel protein concentration, and Z1 and Z2 zein fractions. Within Z2, 15 kDa β-zein explained the largest ethanol yield variation generated by genotypes, N fertilizations, and growth environments. However, and although these differences were as large as 10%, ethanol field yield (L ha^{-1}) was mainly driven by crop yields (r^2 0.98) due to the large crop yield (Mg ha^{-1}) differences observed across treatments. Together, our results helped describe the magnitude that changes in maize kernel hardness can have over ethanol yield, both through genotype selection or crop management. A particular Z2 zein protein rises as relevant for future genetic manipulations of maize ethanol yield determination.

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Maize Kernel Hardness, Endosperm Zein Profiles, and Ethanol Production

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Abstract Maize (*Zea mays* L.) grain is an important feedstock for the ethanol-producing industry. However, little is known about the optimum grain quality for optimizing ethanol yielding efficiencies. We specifically investigated the response of ethanol yields (L Mg^{-1}) to kernel hardness, and its physiological determinant endosperm zein protein profiles, as affected by genotype selection, field nitrogen (N) fertilization, and crop growth environment. We measured ethanol yield and related this to different kernel hardness indicators, kernel composition, and zein profiles. We also described changes in field ethanol yield (L ha^{-1}), by taking into account the crop yield (Mg ha^{-1}). Hard endosperm genotypes always yielded less ethanol than softer endosperm ones per grain mass (L Mg^{-1}). Higher N fertilization rates increased kernel hardness and decreased ethanol yield (L Mg^{-1}) on soft endosperm dented genotypes but had no effect on hard endosperm ones. Ethanol yield was negatively correlated with kernel density, kernel protein concentration, and Z1 and Z2 zein fractions. Within Z2, 15 kDa β-zein explained the largest ethanol yield variation generated by genotypes, N fertilizations, and growth environments. However, and although these differences were as large as 10%, ethanol field yield (L ha^{-1}) was mainly driven by crop yields (r^2 0.98) due to the large crop yield (Mg ha^{-1}) differences observed across treatments. Together, our results helped describe the magnitude that changes in maize kernel hardness can have over ethanol yield, both through genotype selection or crop management. A particular Z2 zein protein rises as relevant for future genetic manipulations of maize ethanol yield determination.

Keywords *Zea mays* L. · Ethanol · Kernel quality · Zein profile · Kernel hardness · Kernel type

Introduction

Maize (*Zea mays* L.) grain is used as feedstock in many industrial processes. One current main destination is the production of ethanol as biofuel. Starch is the major component of the maize kernel (approximately 70 g 100 g^{-1}) and is the basis for this ethanol production process. It yields fermentable sugars that are converted into ethanol [1–3].

Maize ethanol is commonly produced using dry milling. Whole kernels are milled, and endosperm starch is gelatinized and hydrolyzed into dextrins by α-amylase. This process is known as liquefaction. The liquefied slurry is later treated with glucoamylase to yield glucose, which is then fermented into ethanol by yeast (*Saccharomyces cerevisiae*) [1, 3, 4].

Maize ethanol yield is described by the amount of ethanol produced per unit of grain mass (i.e., L Mg^{-1}) but can also take into consideration the amount of ethanol produced per unit of field land area (i.e., L ha^{-1}). The first parameter provides a notion of the economics of the conversion of grain into ethanol [5]. The second parameter is dependent on both

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ethanol yield per unit of grain mass (L Mg^{-1}) and the crop grain yield at the field (Mg ha^{-1}), integrating both grain production and its conversion into ethanol.

Grain conversion into ethanol has been associated with starch structure (amylose content and amylopectin chain length distribution) [3] and protein concentration [6, 7]. It has also been related to kernel density and hardness [8, 9]. In general, there is poor correlation between starch concentration in the kernel and final ethanol yield [6, 8–11], mostly because starch granules are mixed within a protein matrix that interacts.

We are particularly interested in further understanding the connection between kernel hardness and its fermentability. There is a wide range in kernel hardness evident at most maize production regions, and segregating grain for kernel hardness is commonly done by many mills. Our working hypothesis is that maize hybrids with softer endosperms, resulting from genotype selection, environmental conditions, or crop management, will have increased ethanol yield (L Mg^{-1}) resulting from a softer protein matrix that will lead to increased starch conversion into ethanol. We also hypothesize that specific protein fractions responsible for the negative correlation between hardness and fermentability can be detected.

Kernel hardness has traditionally been related to kernel protein concentration, in particular to the content of some specific endosperm proteins called zeins [12–14]. Zeins are alcohol-soluble proteins that may, or may not, require reduction before extraction. They are the main components of the endosperm protein matrix where starch granules are embedded [15]. Zeins accumulate in the endosperm in protein bodies, with α- and δ-zeins (zeins 1, Z1) concentrating in the core of the bodies, and β- and γ-zeins (zeins 2, Z2) on their surface [16]. Figure 1 describes the zein profile from a particular genotype for illustrating the different zein types. Kernel hardness has been particularly correlated to Z2 zeins, the combination of γ- and β-zeins. They are found in greater concentrations in horny endosperm fractions [12, 17, 18] and in genotypes with higher kernel hardness [19]. Ubach et al. [20] called "low-fermentability corn" to that having high concentrations of 15 kDa β-zein (C zein), 16 kDa γ-zein (F zein), and 19 kDa α-zein (a component of the Z1 fraction), while maize holding low concentrations of these particular zeins were classified as "high-fermentability corn." However, these conclusions were mostly narrated, without experimental evidences. They did not test the specific effect of any crop management practice or environment on these parameters and ethanol yield nor discriminated genotype differences at these specific zeins for understanding the magnitude or their effect in ethanol yield.

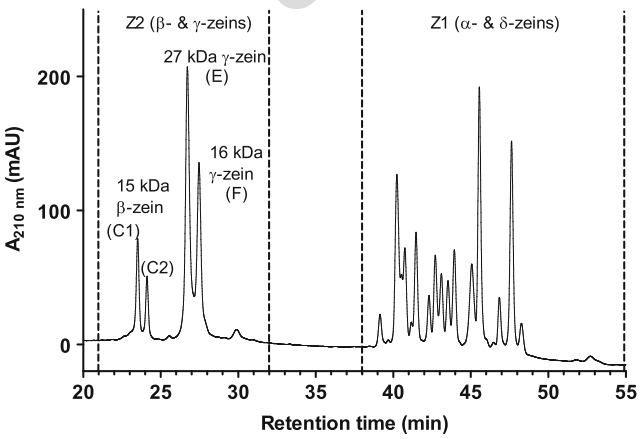

Fig. 1 Chromatogram describing the different zein types of a maize kernel. It corresponds to one replicate of genotype ACA530 (exp. 2). The *y* axis represents the absorbance at $\lambda = 210$ nm (mAU) and the *x* axis represents the retention time (min)

Nitrogen fertilization is a common management practice and has a direct effect over the crop yield, kernel protein concentration [21–23], and kernel hardness [24]. Nitrogen fertilization affects zein concentration in the endosperm under both field [19, 21, 25, 26] and in vitro [27] growing conditions, but its direct effect over grain fermentability is not known.

We are interested in understanding maize kernel fermentability and ethanol yield, specifically as related to kernel hardness. Our objectives were (i) to test how genotype differences in kernel hardness are affecting ethanol yield, (ii) to assess the effect of N fertilization over kernel hardness and ethanol yield, and (iii) to understand the relationships between maize kernel hardness, kernel composition (protein, starch, and oil), endosperm zein profiles, and ethanol production.

Materials and Methods

Two field experiments were conducted at Facultad de Ciencias Agrarias in Zavalla, Santa Fe, Argentina (33° 1′ S, 60° 53′ W). Each experiment was replicated during two environmental conditions.

Exp. 1: Genotypes with Contrasting Kernel Hardness and N Fertilization Treatments

A fermentation study was completed as part of a physicochemical quality test of commercial maize hybrids as impacted by N fertilization [19]. Four commercial genotypes and three levels of N application were combined in a completely randomized block design with three replicates. The experiment was conducted during two growing seasons (2012/2013 and 2013/2014). Genotypes included two regular soft endosperm dents (DK747 and AX887) and two hard endosperm flints (ACA2002 and NT426). Genotypes represent widely planted genotypes for both kernel types at the central Argentinean region during the timing of the experiment. Planting dates were September 21 (2012) and October 2 (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 pl m^{-2} was used across treatments, and plots were overplanted and hand-thinned soon after emergence. Plots were managed following common agronomic practices for the region for weeds and diseases and grown under rainfed conditions. Because genotypes were different for insect resistance, we carefully controlled any insect presence with recommended products throughout the growing seasons.

At planting, monoammonium phosphate (MAP, 10-50-0, N-P-K) was applied at a rate of 120 kg ha^{-1} for all plots, and after this, three N treatment levels were arranged: (i) a low N treatment, (ii) an intermediate N treatment, and (iii) a high N one. Soil samples (0–60 cm) were taken before planting and analyzed for N-NO_3 (0–60 cm), and N was applied as urea (46-0-0, N-P-K) for reaching three fertilization levels. In the low N level, no N other than MAP was used. At this treatment, the soil reached, on average across years, 85 kg N ha^{-1} (N from the soil at planting plus N from MAP). At the intermediate level, urea was applied for adjusting the soil N level to 155 kg N ha^{-1} (N from the soil at planting plus MAP plus urea). At the high N level, the soil was adjusted for reaching 250 kg N ha^{-1} (N from the soil at planting plus MAP plus urea). The urea was broadcasted over the plots soon after thinning (V3–V4). At commercial maturity, two central rows per plot were manually harvested and shelled with a mechanical thresher. Yield was calculated and presented on a 14.5% moisture basis.

Exp. 2: Genotypes with Contrasting Kernel Hardness

This experiment was conducted to further test the importance of genotype kernel hardness differences. Twenty-three commercial genotypes with contrasting hardness (ACA2002, ACA2002Bt, ACA514, ACA530, AX7822, CyR7325, DA-648, DK692, DK7210, EG806, EG807, EG808, Exp-032, Mil522, AX8010, NK940, NK960Bt, NT426, NT426Bt, NT525, NT525Bt, P1780, and SPS2866) were combined in a completely randomized block design with three replicates. Environmental replication was done by using contrasting planting dates, September 29 (2014) and December 18 (2014).

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 7.5 pl m^{-2} was used across treatments, and plots were overplanted and hand-thinned. Plots were managed following common agronomic practices for the region for weeds and diseases and grown under rainfed conditions. Similar to exp. 1, because genotypes were different for insect resistance, any insect presence was controlled with recommended products throughout the growing season. At commercial maturity, the experiment was harvested and processed similarly to exp. 1.

Kernel Hardness

Kernel hardness was evaluated with test weight and kernel density. Test weight was measured using a homogeneous grain aliquot determined with a Schopper chondrometer and expressed as kilograms per hectoliter. Kernel density was measured using 20 intact, non-damaged maize kernels. They were placed into a 50-mL burette containing 20 mL ethanol. Air bubbles were removed. Alcohol volume difference before and after the addition of kernels was recorded. The kernels were dried in a forced air oven at 65 °C for 96 h and their weight was recorded. Kernel density (g cm^{-3}) was calculated as the ratio between dry kernel weight (g) and kernel volume (cm^3) [19, 28].

Kernel Composition and Zein Profiles

Protein, starch, and oil percentages were determined by near-infrared spectroscopy using an Infratec 1241 instrument (Foss, Denmark). Results were expressed on dry basis.

Zein profiles were determined by HPLC [29]. An aliquot of 100 kernels was ground in a laboratory grinder (Tecnodalvo, Buenos Aires, Argentina). The flour (200 mg) was defatted twice with 1 mL hexane for 1 h under agitation (140 rpm). The mixture was centrifuged at 12,000×*g* and 5 °C for 10 min after each extraction and the supernatant was discarded. The pellet was left overnight under the extraction hood for residual hexane evaporation. Zeins were extracted with 1 mL of 70% ethanol containing 5% β-mercaptoethanol and 0.5% sodium acetate at room temperature for 2 h and 140 rpm agitation. The mixture was centrifuged at 12,000×*g* and 5 °C for 10 min and an aliquot of the supernatant was diluted 1:5 with the extraction solvent and filtered through a 0.22-μm pore nylon syringe filter. The filtrate was injected in a Dionex Ultimate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA) equipped with a 4.6 × 250 mm 218MS 300 Å pore size Vydac C18 column (Grace Davison Discovery Sciences, Deerfield, IL) at 60 °C. The mobile phase system was acetonitrile (solvent A) and water (solvent B) both containing 0.10% trifluoroacetic acid (TFA). 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 1 mL min^{-1}, and UV detection was set at 210 nm [17].

For zein peak identification, B57, N28, A619, and W64A genotypes from the USDA Germplasm Bank were run and compared to the results of Wilson [15] and Eyhérabide et al. [17] (Fig. 1). Peak quantification was done using peak area (mAU min) relative to the mass (g, dry basis) of extracted maize [30]. Moisture content of the flour was determined by weight difference of a 2-g aliquot dried in an oven for 2 h at 130 °C.

Ethanol Yield Determination

Maize kernels were milled using a Loyto #1 hammer mill (Loyto, Argentina) equipped with a 2-mm screen. A 10-g meal aliquot was dispersed in tap water to obtain a slurry with 20% solids (final concentration) in a 50-mL centrifuge tube. The pH was adjusted to 5.6 to 5.8 with 0.1 M NaOH or H_2SO_4. Enough alpha amylase (Liquozyme, Novozymes, USA) was added to have a relationship of 0.25 kg enzyme per Mg of meal solids. The capped tubes were shaken horizontally during 120 min at 85 °C in a water bath at 160 rpm. The liquefied product was cooled to 30 °C and the pH adjusted to 5.0 to 5.2. Enough glucoamylase was added to obtain 0.50 kg enzyme per Mg of solids. Urea and virginiamycin (Lactrol, Phibro, USA) were added to yield 500 and 2 ppm, respectively. Ethanol red yeast (Lessafre, USA) was dispersed in tap water (1.35 g per 100 mL) at 35 °C and kept at this temperature for 15 min before adding 1 mL of yeast suspension to each tube. The tubes were vortex-mixed and incubated loosely capped in oven at 32 °C for 72 h. At 24 and 48 h of incubation, the tubes were vortex-mixed. After 72 h incubation, 100 μL concentrated H_2SO_4 was added to stop glucoamylase, and a beer aliquot was centrifuged at 14,000×*g* and 5 °C for 10 min. Supernatant was diluted 1:5 with deionized water and filtered through a 0.22-μm nylon syringe filter. The filtrate (20 μL) was injected in a Dionex 3000 HPLC system (Thermo Dionex, USA) equipped with an Aminex HPX-87H column running isocratically on 0.025 M H_2SO_4 at 0.6 mL min^{-1}. The column was held at 60 °C and a Refractomax 520 refractive index detector (ERC, Germany) was used at 50 °C for detection. Ethanol was quantified using a five-point calibration curve prepared from dilutions of a fuel ethanol residual saccharide mix (Supelco, USA).

Ethanol yield was calculated as volume (L) ethanol per mass (Mg) dry maize considering 0.79 kg L^{-1} ethanol density. We also calculated an ethanol field yield, as volume ethanol per land area unit (L ha^{-1}).

Statistical Analysis

Experiments 1 and 2 were analyzed separately. Results were analyzed by analysis of variance (ANOVA) using PROC GLM from SAS (SAS Institute, Cary, NC). In exp. 1, the model included kernel type (flint or dent), genotypes nested within kernel type, N treatment, year, and their interactions. In exp. 2, the model included genotypes, environment, and their interaction. In exp. 1, the four genotypes were selected for their contrasting hardness, and they could be easily discriminated between dent and flint kernel type. In exp. 2, this could not be done, as genotypes showed a kernel hardness continuum, from dents, to semi-dents, and flints. In both experiments, factors were all considered as fixed effects. The level of significance was established at $P < 0.05$, except when mentioned, and least significant differences (LSD) were calculated. Percentage sum squares (% SS) were calculated to estimate the contribution of each effect to total variation. Pearson correlation coefficients between kernel composition, kernel physical parameters, Z1, Z2, its components (15 kDa β-zeins or C zeins, 27 kDa γ-zein or E zein, and 16 kDa γ-zein or F zein), and ethanol yield (L Mg^{-1}) were determined using PROC CORR from SAS.

Results

Grain Yield and Kernel Hardness

Field grain yield is an important component of the ethanol production process because farmers are paid per megagram of grain produced. It is also relevant for the calculation of ethanol production on a land area basis (L ha^{-1}). Yields from exp. 1 ranged from 2.14 to 13.82 Mg ha^{-1}, and the effects of kernel type (dent vs. flint), genotype within kernel type, year, and N fertilization are described in Table 1. In brief, kernel type and N fertilization significantly affected yield. Higher N availability increased grain yield, and dented genotypes yielded more than flint ones (Table 1). Dryer and warmer conditions observed in year 2 resulted in quite lower grain yields than year 1 [19]. Details may be observed in Supplementary Material 1.

In exp. 2, yields ranged from 9.16 to 15.73 Mg ha^{-1} ($P < 0.01$; Table 2). Environment accounted for most yield variability (50% SS), followed by genotype (44% SS), and the significant environment × genotype interaction (6% SS). DK7210 and AX7822 were the top-yielding genotypes in the early environment, while in the later one, they shared the highest yields with DK692, NT426BT, and NT525BT (Supplementary Material).

In exp. 1, flint kernel type had higher hardness (test weight and kernel density) than dent kernel type ($P < 0.05$). Increasing N fertilization also increased test weight and kernel density for both kernel types (Table 1).

Significant differences in test weight were observed across genotypes and environments in exp. 2. The interaction environment × genotype was also significant for test weight ($P < 0.05$; Table 2); however, most variability was related to genotype differences (95% SS). Exp-032 and DA-648 genotypes had the highest test weight values at both environments. Genotypes also showed differences in kernel density in exp. 2 ($P < 0.001$), ranging from 1.140 to 1.229 g cm^{-3}.

Kernel Composition

In exp. 1, starch concentration in kernels was only affected by kernel type ($P < 0.001$), where dents had consistently higher starch concentrations than flint ones. Dented genotypes had

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t1.1 **Table 1** Description of grain yield, test weight, kernel density, and composition of two flint (ACA2002, NT426) and dent (AX887, DK747) kernel type genotypes grown at three nitrogen fertilization levels for 2 years (exp. 1). The description of every particular combination of year × N treatment × genotype is available as Supplementary Material Q6

t1.2	Year	Kernel type	Genotype	N treatment	Grain yield[a]	Test wt.[a]	Kernel density[a]	Starch	Protein[a]	Oil
t1.3					Mg ha^{-1}	kg hL^{-1}	g cm^{-3}	g 100 g^{-1}		
t1.4	Year 1				10.36	77.9	1.165	67.2	8.8	5.0
t1.5	Year 2				5.24	78.6	1.205	68.2	11.1	4.9
t1.6		Dent			8.95	77.9	1.166	68.9	9.5	4.5
t1.7		Flint			6.46	78.7	1.204	66.5	10.4	5.3
t1.8			AX887		8.97	78.1	1.173	69.5	9.4	4.5
t1.9			DK747		8.94	77.7	1.158	68.3	9.6	4.5
t1.10			ACA2002		5.45	78.0	1.194	66.8	10.8	5.0
t1.11			NT426		7.44	79.2	1.214	66.1	9.9	5.6
t1.12				Low	6.90	77.9	1.177	68.5	9.0	4.8
t1.13				Intermediate	7.98	78.4	1.181	67.3	10.2	4.9
t1.14				High	8.29	78.4	1.197	67.2	10.6	5.0
t1.15	Year (Y)				***	***	***	–	***	*
t1.16	Kernel type (KT)				(0.46)***	***	***	(1.4)***	***	***
t1.17	Genotype (kernel type)				***	***	*	–	***	***
t1.18	Nitrogen (N)				***	*	*	–	(0.2)***	**
t1.19	Y × KT				–	(0.4)**	–	–	(0.3)**	–
t1.20	Y × genotype (kernel type)				(0.92) *	–	(0.026)*	–	(0.4)***	(0.1)**
t1.21	KT × N				–	–	–	–	–	(0.1)**
t1.22	Y × N				(0.79)***	–	–	–	–	(0.1)*
t1.23	N × genotype (kernel type)				–	(0.8)**	–	–	–	(0.2)*
t1.24	Y × KT × N				–	–	(0.032)*	–	–	–
t1.25	Y × N × genotype (kernel type)				–	–	–	–	–	–

Numbers in parentheses represent the least significant differences (LSD) of the means

*Significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$

[a] From Gerde et al. [19]

on average across all treatments, genotypes, and experimental years 68.9 g 100 g^{-1} starch, while flint kernel type had 66.5 g 100 g^{-1} (Table 1). Nitrogen fertilization had no effect over kernel starch concentration. In exp. 2, the concentration of starch in the kernels was significantly affected by genotypes only ($P < 0.001$), with values averaged across environments ranging from 69.1 to 72.5 g 100 g^{-1} (Table 2).

Significant differences in protein concentration were observed between kernel types in exp 1. Flint kernel type had significantly more kernel protein concentration than dents (10.4 and 9.5 g 100 g^{-1}, respectively), and genotype differences within each kernel type were evident ($P < 0.001$, Table 1). Increasing N fertilization resulted in consistently higher protein concentrations. Kernel protein concentration values during year 2 were significantly higher than those during year 1, and flint kernel types increased their protein concentration more than dents.

In exp. 2, significant differences in protein concentration were observed for genotypes and environments ($P < 0.001$; Table 2). There was also a significant genotype × environment interaction ($P < 0.05$; Table 2), but genotype differences accounted for most variation (84% SS).

Kernel oil concentration showed significant year, kernel type, genotype within kernel type, and N fertilization effects in exp. 1 ($P < 0.05$). Genotype within kernel type accounted for most variation (90% SS), with both flint kernel type genotypes having higher oil concentrations than dents. Also, year × genotype within kernel type, kernel type × N fertilization, and year × N fertilization interactions were significant ($P < 0.05$; Table 1) but explained small portions of the total explored variability (less than 5% SS).

In exp. 2, genotypes and environments affected the kernel oil concentration (Table 2). As in exp. 1, genotypes accounted for most variation (97% SS).

Zein Profiles

In exp. 1, the concentration of Z1 (the combination of α- and δ-zeins, Fig. 1) was affected mainly by the N fertilization treatment (27% SS), as reported by Gerde et al. [19], with

Table 2 Description of grain yield, test weight, kernel density, and composition of 23 genotypes grown in two environments (early and late planting, exp. 2). Mean values of every particular environment × genotype combination are available as Supplementary Material

Treatment		Grain yield	Test wt.	Kernel density	Starch	Protein	Oil
		Mg ha^{-1}	kg hL^{-1}	g cm^{-3}	g 100 g^{-1}		
Environment	Early	13.21	79.4	1.182	71.4	9.0	5.0
	Late	10.79	79.7	1.186	71.2	8.6	5.1
Genotype	AX7822	14.19	76.5	1.141	72.4	8.1	4.4
	DK692	13.47	78.0	1.140	72.5	8.0	4.7
	DK7210	14.81	77.4	1.134	72.2	7.8	4.6
	NK960Bt	12.85	79.1	1.175	70.0	9.1	5.7
	P1780	12.76	77.5	1.144	72.3	8.2	4.3
	ACA2002	11.24	80.0	1.189	71.4	9.4	5.0
	ACA2002Bt	10.97	79.3	1.188	71.4	9.4	4.9
	ACA514	12.21	80.0	1.179	71.7	8.6	4.7
	ACA530	10.76	80.7	1.229	70.3	9.9	5.2
	CyR7325	11.63	79.8	1.200	71.7	8.8	4.8
	DA-648	10.77	82.5	1.199	69.6	9.7	5.9
	EG806	10.79	79.8	1.186	71.7	8.7	5.0
	EG807	11.93	80.1	1.199	71.9	8.7	4.8
	EG808	11.52	79.8	1.195	71.2	8.7	5.1
	Exp-032	10.57	82.5	1.203	69.1	9.8	6.3
	Mil522	11.25	80.4	1.206	71.2	9.4	4.9
	NK940	11.80	78.6	1.185	70.7	8.9	5.4
	NT426	12.36	79.5	1.202	70.8	8.8	5.5
	NT426Bt	13.21	80.2	1.203	70.8	8.9	5.6
	NT525	12.21	80.2	1.205	72.9	8.1	4.7
	NT525Bt	13.15	79.5	1.154	72.4	7.9	4.7
	AX8010	12.77	79.5	1.196	71.2	9.1	4.9
	SPS2866	12.77	78.3	1.172	70.8	8.7	5.4
Environment (E)		***	**	–	–	***	(0.1)**
Genotype (G)		***	***	(0.039)***	(0.6)***	***	(0.2)***
E × G		(1.15)**	(0.8)*	–	–	(0.5)*	–

Numbers in parentheses represent the least significant differences (LSD) of the means

*Significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$

increasing Z1 at increasing N fertilization. The concentration of Z2 (the combination of β- and γ-zeins) was mostly dependent on the kernel type (73% SS), with significantly higher values for the flint genotypes. Although several interactions were also significant in determining the concentration of Z1 and Z2 (Table 3), they accounted for minor variation portions.

Experiment 2 explored more genotypes than exp. 1, and significant effects over Z1 were genotype ($P < 0.001$), environment, and the interaction genotype × environment ($P < 0.05$; Table 4). Genotype accounted for most variation (89% SS), followed by environment × genotype (10% SS). The interaction was mostly related to genotypes exploring larger Z1 concentrations in the late environment than in the earlier one (Supplementary Material).

Genotype, environment, and environment × genotype had significant effects ($P < 0.001$) in the concentration of Z2 in exp. 2. Similar to Z1, genotype differences accounted for most variation (94% SS). Environment and environment × genotype effects represented smaller portions of Z2 variation (2 and 4% SS, respectively). Averaged across environments, minimum Z2 concentrations were observed in genotypes DK7210 (685 mAU min g^{-1}) and AX7822 (743 mAU min g^{-1}), while DA-648 had the highest concentration (1358 mAU min g^{-1}).

We further explored the concentration of specific Z2 components, as described in Fig. 1. In exp. 1, differences were observed for both 15 kDa β-zein subclasses, C1 and C2. Significant differences were observed in C1 for year, kernel type, genotype within kernel type ($P < 0.001$), N fertilization ($P < 0.05$), and the interactions year × kernel type ($P < 0.001$) and year × genotype ($P < 0.01$; Table 3). In addition to the significant factors observed in C1, significant differences ($P < 0.05$) were observed in C2 for kernel type × N fertilization, year × kernel type × N fertilization ($P < 0.05$), N fertilization × genotype, and year × N fertilization × genotype

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t3.1 **Table 3** Description of the concentrations of Z1, Z2, 15 kDa β-zein (C1 and C2), and 27 kDa (E) and 16 kDa (F) γ-zeins of two flint (ACA2002, NT426) and dent (AX887, DK747) kernel type genotypes grown at three nitrogen fertilization levels for 2 years (exp. 1). Zein concentrations are expressed as peak area units relative to mass of maize (mAU min g^{-1}). The description of every particular combination of year × N treatment × genotype is available as Supplementary Material

	Year	Kernel type	Genotype	N treatment	Z1 peak group[a]	Z2 peak group[a]	15 kDa β-zein (C1)	15 kDa β-zein (C2)	27 kDa γ-zein[a] (E)	16 kDa γ-zein (F)
t3.3					mAU min g^{-1}					
t3.4	Year 1				1649	777	44	31	380	196
t3.5	Year 2				2044	889	63	55	338	185
t3.6		Dent			1646	649	15	81	237	179
t3.7		Flint			2047	1012	89	6	480	202
t3.8			AX887		1588	625	4	93	228	157
t3.9			DK747		1704	671	26	69	247	200
t3.10			ACA2002		2261	968	84	8	438	194
t3.11			NT426		1833	1057	94	5	523	210
t3.12				Low	1585	799	52	36	349	189
t3.13				Intermediate	1795	793	49	43	327	175
t3.14				High	2160	904	57	50	402	208
t3.15	Year (Y)				***	***	***	***	***	**
t3.16	Kernel type (KT)				***	***	***	***	***	***
t3.17	Genotype (kernel type)				***	***	***	***	***	***
t3.18	Nitrogen (N)				***	***	(7)*	***	***	***
t3.19	Y × KT				***	***	(8)***	***	***	–
t3.20	Y × genotype (kernel type)				(177)*	***	(11)**	***	**	–
t3.21	KT × N				–	–	–	*	–	***
t3.22	Y × N				**	**	–	–	***	***
t3.23	N × genotype (kernel type)				–	–	–	***	***	**
t3.24	Y × KT × N				(217) ***	(72)**	–	(9)*	(30)***	(17)*
t3.25	Y × N × genotype (kernel type)				–	(102) ***	–	(13)***	(43)***	(25)*

Numbers in parentheses represent the least significant differences (LSD) of the means

*Significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$

[a] From Gerde et al. [19]

interactions ($P < 0.01$; Table 3). In both cases, kernel type accounted for most variation with 78 and 72% SS for C1 and C2, respectively. It is important to note that while C1 was the predominant 15 kDa β-zein in flint genotypes, C2 was the most abundant β-zein subclass in AX887, a dent kernel type genotype. DK747, the other dent type genotype, had equivalent concentrations of C1 and C2 during year 1 and C2 was more abundant than C1 during year 2 (Table 3). Nitrogen fertilization contributed to increased concentrations of the predominant β-zein (C1 or C2) in exp. 1.

Within 15 kDa β-zein in exp. 2, the concentration of the C1 subclass was affected by genotype only ($P < 0.001$; Table 4). The maximum values corresponded to Exp-032, DA-648, and NT426 (172, 168, and 158 mAU min g^{-1}, respectively), while minimums corresponded to DK692 and P1780 (9 and 11 mAU min g^{-1}, respectively). Genotype ($P < 0.001$) and environment ($P < 0.01$) significantly impacted on the concentration of C2 β-zein subclass, with genotype accounting for most variation (98% SS). The concentration of C2 was highest in DK692 and P1780 (113 and 107 mAU min g^{-1}, respectively). A small, but significant, higher C2 was observed in the later planting environment (Table 4).

In exp. 1, the concentration of 27 kDa γ-zein (E zein) was significantly affected ($P < 0.01$) by all factors and interactions, except kernel type × N fertilization (Table 3). Kernel type accounted for most variation (73% SS), being the concentration of 27 kDa γ-zein higher in flint kernel type genotypes.

In exp. 2, significant effects ($P < 0.001$; Table 4) were observed in 27 kDa γ-zein for genotype (92% SS),

Table 4 Description of the concentrations of Z1, Z2, 15 kDa β-zein (C1 and C2), and 27 kDa (E) and 16 kDa (F) γ-zeins of 23 genotypes grown in two environments (early and late planting, exp. 2). Zein concentrations are expressed as peak area units relative to mass of maize (mAU min g^{-1}). Values of every particular combination of environment × genotype within kernel type are presented as Supplementary Material

Treatment		Z1 peak group	Z2 peak group	15 kDa β-zein (C1)	15 kDa β-zein (C2)	27 kDa γ-zein (E)	16 kDa γ-zein (F)
		mAU min g^{-1}					
Environment	Early	1648	1013	104	35	493	245
	Late	1593	1067	103	40	546	255
Genotype	AX7822	1439	743	33	70	331	206
	DK692	1317	862	9	113	381	233
	DK7210	1196	685	51	54	277	221
	NK960Bt	1586	1083	121	13	573	259
	P1780	1381	891	11	107	447	215
	ACA2002	1896	953	95	17	492	256
	ACA2002Bt	1879	1039	112	15	525	260
	ACA514	1695	860	140	10	397	189
	ACA530	2211	1028	117	56	470	269
	CyR7325	1518	1204	82	67	639	264
	DA-648	1972	1358	168	14	708	281
	EG806	1696	880	139	9	373	258
	EG807	1617	1197	78	76	636	267
	EG808	1868	919	139	13	386	261
	Exp-032	2017	1303	172	12	689	284
	Mil522	1964	1227	147	10	657	245
	NK940	1481	1165	136	16	599	269
	NT426	1547	1192	158	11	598	290
	NT426Bt	1498	1179	148	16	591	282
	NT525	1353	1072	63	60	564	231
	NT525Bt	1167	1001	65	56	505	229
	AX8010	1622	914	80	14	484	222
	SPS2866	1419	1100	114	26	568	251
Environment (E)		*	***	–	(4)**	***	(5)***
Genotype (G)		***	***	(17)***	(13)***	***	(17)***
E × G		(217) ***	(92)***	–	–	(51)***	–

Numbers in parentheses represent the least significant differences (LSD) of the means

*Significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$

environment (5% SS), and the genotype × environment interaction (3% SS). Highest concentrations of 27 kDa γ-zein were observed in DA-648 and Exp-032 in both environments (Supplementary Material).

Significant effects ($P < 0.05$) were observed in the concentration of 16 kDa γ-zein (F zein) for most treatment factors and interactions in exp. 1 (Table 3). In contrast with 15 and 27 kDa zeins, variation for the 16 kDa zein was more evenly distributed among variation sources, with genotype within kernel type, N fertilization, kernel type, and the interaction year × N fertilization accounting for 26, 18, 14, and 18% SS, respectively. The N fertilization effect over this particular zein was not clear.

In exp. 2, the concentration of 16 kDa γ-zein was also significantly influenced by genotype (91% SS; $P < 0.001$) and environment ($P < 0.01$). NT426, NT426Bt, Exp-032, and DA-648 had the highest concentrations of 16 kDa γ-zein with 290, 284, 281, and 281 mAU min g^{-1}, respectively (Table 4).

Ethanol Yields

Ethanol yield (L Mg^{-1}) in exp. 1 showed significant year, kernel type ($P < 0.001$), and N fertilization ($P < 0.05$) main effects (Table 5). Significant interactions ($P < 0.05$) were year × kernel type and N fertilization × genotype within kernel type. The year accounted for most variation (45% SS) with a mean yield of 376 L Mg^{-1} for year 1 and 362 L Mg^{-1} for year 2, averaged across treatments. Kernel type was also highly

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t5.1 **Table 5** Ethanol yield of two flint (ACA2002 and NT426) and two dent (AX887, DK747) kernel type genotypes grown at three N fertilization levels for 2 years (exp. 1). The description of every particular combination of year × N treatment × genotype is available as Supplementary Material

	Year	Kernel type	Genotype	N treatment	Ethanol yield	
t5.3					L Mg^{-1}	L ha^{-1}
t5.4	Year 1				376	3186
t5.5	Year 2				362	1623
t5.6		Dent			373	2795
t5.7		Flint			365	2014
t5.8			AX887		373	2815
t5.9			DK747		372	2775
t5.10			ACA2002		368	1776
t5.11			NT426		362	2252
t5.12				Low	373	2198
t5.13				Intermediate	369	2491
t5.14				High	365	2525
t5.15	Year (Y)				***	***
t5.16	Kernel type (KT)				***	(173)***
t5.17	Genotype (kernel type)				–	***
t5.18	Nitrogen (N)				*	**
t5.19	Y × KT				(6)*	–
t5.20	Y × genotype (kernel type)				–	(346)**
t5.21	KT × N				–	–
t5.22	Y × N				–	(300)***
t5.23	N × genotype (kernel type)				(10)*	–
t5.24	Y × KT × N				–	–
t5.25	Y × N × genotype (kernel type)				–	–

Numbers in parentheses represent the least significant differences (LSD) of the means

*Significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$

t6.1 **Table 6** Ethanol yield of 23 genotypes grown in two environments (early and late planting, exp. 2). Values of every particular combination of environment × genotype are presented as Supplementary Material

Treatment		Ethanol yield		
		L Mg^{-1}	L ha^{-1}	
Environment	Early	377	4255	t6.4
	Late	385	3552	t6.5
Genotype	AX7822	390	4730	t6.6
	DK692	380	4366	t6.7
	DK7210	382	4823	t6.8
	NK960Bt	388	4261	t6.9
	P1780	384	4182	t6.10
	ACA2002	379	3638	t6.11
	ACA2002Bt	370	3463	t6.12
	ACA514	382	3989	t6.13
	ACA530	363	3347	t6.14
	CyR7325	382	3746	t6.15
	DA-648	377	3466	t6.16
	EG806	376	3477	t6.17
	EG807	380	3871	t6.18
	EG808	390	3832	t6.19
	Exp-032	373	3374	t6.20
	Mil522	378	3627	t6.21
	NK940	372	3736	t6.22
	NT426	379	4048	t6.23
	NT426Bt	377	4261	t6.24
	NT525	384	4005	t6.25
	NT525Bt	391	4362	t6.26
	AX8010	385	4184	t6.27
	SPS2866	382	4165	t6.28
Environment (E)		(2)***	***	t6.29
Genotype (G)		(7)***	***	t6.30
E × G		–	(391)*	t6.31

Numbers in parentheses represent the least significant differences (LSD) of the means

*Significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$

significant, accounting for 14% SS, with dents showing higher yields (373 vs. 365 L Mg^{-1}, for dents and flints, respectively).

Increased crop N fertilization decreased ethanol yields (373, 369, and 365 L Mg^{-1} for low, intermediate, and high N fertilization, respectively). However, the effect was not similar for all genotypes (significant N fertilization × genotype within kernel type interaction, $P < 0.05$). No differences ($P > 0.05$) were observed for flint genotypes (ACA2002 and NT426) across N treatments, but dent genotypes (AX887 and DK747) yielded less ethanol whenever N fertilization was increased ($P < 0.05$; Table 5).

In exp. 2, ethanol yield (L Mg^{-1}) was significantly affected by genotype and environment ($P < 0.001$; Table 6). Genotype differences accounted for more variation (62% SS) when compared to the environment (24% SS). The highest ethanol-yielding genotypes were NT525Bt, AX7822, EG808, NK960Bt, AX8010, P1780, and NT525 with 391, 390, 390, 388, 385, 384, and 384 L Mg^{-1}, respectively. The later planting environment yielded, on average, 8 L Mg^{-1} more than the earlier one (Table 6).

In exp. 1, field ethanol yields (L ha^{-1}) showed significant ($P < 0.01$) year, kernel type (dent or flint), genotype within kernel type, and N fertilization main effects (Table 5). Significant interactions ($P < 0.01$) were year × genotype within kernel type and year × N fertilization. Year accounted for most of the variation (66% SS) with average field ethanol yields of 3186 L ha^{-1} during the first year and 1623 L ha^{-1} during the second one. Kernel type also explained important part of the variation (17% SS), with 2795 L ha^{-1} for dent kernel type and 2014 L ha^{-1} for flint kernel type. Genotypes

within each kernel type were significantly different, especially when flint genotypes are compared (1776 and 2252 L ha^{-1} for ACA2002 and NT426, respectively; Table 5). Nitrogen fertilization effect was also significant ($P < 0.01$); N fertilization rates increased field ethanol yields when averaged across genotypes and years.

In exp. 2, significant differences were observed in field ethanol yield for genotypes, environments, and the environment × genotype interaction ($P < 0.05$; Table 6). Genotype accounted for most variation (54% SS), followed by environment (40% SS) and the interaction (6% SS). For the early planting environment, AX7822, DK7210, and AX8010 yielded the highest field ethanol, with 5081, 5046, and 4693 L ha^{-1}, respectively. For the late planting environment, the highest field ethanol-yielding genotypes were DK7210, AX7822, NT525Bt, and DK692, with 4526, 4263, 4209, and 4166 L ha^{-1}, respectively (Supplementary Material).

It is relevant to point out that field ethanol yield is the result of the combination of crop grain yield (Mg ha^{-1}) and ethanol yield per grain mass (L Mg^{-1}). As evident in Fig. 2, the field grain yield component explained most variations in field ethanol yield across genotypes and environments.

Ethanol Yield as Related to Kernel Hardness, Composition, and Zeins

Mean values of the year × N fertilization × genotype within kernel type interaction in exp. 1 and genotype × environment in exp. 2 for ethanol yield (L Mg^{-1}) were correlated to kernel hardness indicators and different zein concentrations in order to determine the relationships between them (Table 7).

In exp. 1, ethanol yield (L Mg^{-1}) was significantly ($P < 0.05$) and negatively correlated with test weight, vitreousness, kernel density, and the concentrations of protein, oil, Z1, and Z2. In particular, among Z2, ethanol yield was only significantly and negatively correlated with total concentration of 15 kDa β-zein (C1 + C2) and C1.

In exp. 2, the Z1 zein fraction was negatively correlated with ethanol yield (L Mg^{-1}), while total Z2 was not. When considering each main component of Z2, the 15 kDa β-zein fraction (C1 + C2) was negatively correlated with ethanol yield, in particular the C1 subclass, the predominant β-zein in hard endosperm flint kernel types. The most abundant 27 kDa γ-zein was not correlated with ethanol yield (L Mg^{-1}), but only the 16 kDa γ-zein (Table 7).

Together, results indicated that ethanol yield (L Mg^{-1}) was negatively correlated to kernel hardness. Kernel protein concentration and zeins appear as the main drivers of this negative correlation. Increased kernel hardness through genotype or N fertilization levels showed reductions in ethanol yield. Results also indicated that the kernel starch concentration, the main sugar feedstock for the fermentation process, was only partly related to ethanol yield.

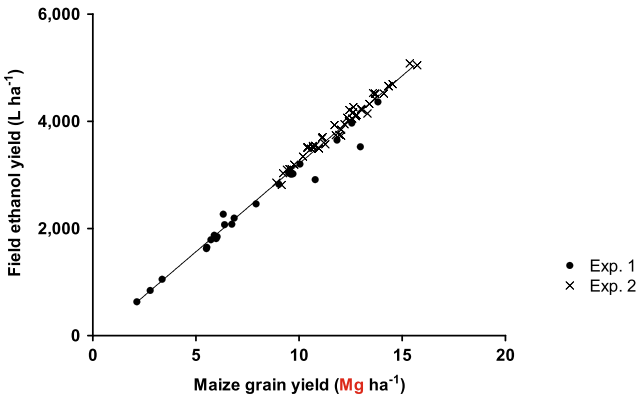

Fig. 2 Relationship between maize grain yield (Mg ha^{-1}) and ethanol yield on a land area basis (L ha^{-1}) for all genotypes and treatments from exps. 1 (•) and 2 (×). Linear regression combining both experiments is $Y = 328X - 76$ (r^2 0.98; n 70; $P < 0.001$)

Discussion

Our results have shown that increased kernel hardness, through genotype, N fertilization management, or crop growth environment, always decreased ethanol yield per unit of grain mass (L Mg^{-1}). And, although these differences can be as large as 10% (Tables 5 and 6), field ethanol yield (L ha^{-1}) was mainly driven by crop yields (Mg ha^{-1}). This is similar to previous studies in barley and wheat [5]. Genotype and environmental differences in field grain yield (Mg ha^{-1}) were much higher than differences in ethanol yield per unit of grain mass. Nitrogen fertilization increased field ethanol yields (L ha^{-1}), even though N fertilization increased kernel hardness and reduced ethanol yield per grain mass (L Mg^{-1}). The reduced ethanol yield due to higher kernel hardness when fertilizing with N was mostly insignificant when compared to the changes observed in the crop field grain yield.

This is not the first study reporting changes in ethanol yield as related to kernel hardness. Several previous reports agree that maize with harder and denser kernels yield less ethanol [8, 9]. However, we are the first to report the specific changes in endosperm characteristics behind differences in ethanol yields. Changes in zein profiles, through genotype selection, N fertilization, or environmental growth environment, affected kernel hardness, ultimately impacting ethanol yields. We are also the first to describe the maximum ethanol yield magnitude (~10%) that can be exploited when segregating among commercial germplasm for kernel hardness. Also, our results showed N fertilization impact on ethanol yields was genotype-dependent (significant genotype within kernel type × N fertilization over ethanol yield; Table 5),

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t7.1 **Table 7** Pearson correlation coefficients (r) between several kernel hardness and composition traits and ethanol yield (L Mg^{-1}) in exps. 1 and 2. In exp. 1, correlations include four genotypes, three fertilization treatments, and two environments (n 24). In exp. 2, correlations include 23 genotypes and two environments (n 46)

	Exp. 1		Exp. 2	
Trait	n	Ethanol yield (L Mg^{-1})	n	Ethanol yield (L Mg^{-1})
Test weight	24	−0.49*	46	n.s.
Kernel density	24	−0.72***	46	−0.40**
Starch	24	n.s.	46	0.35*
Protein	24	−0.78***	46	−0.68***
Oil	24	−0.46*	46	n.s.
Z1 peak group	24	−0.64***	46	−0.52***
Z2 peak group	24	−0.60**	46	n.s.
15 kDa β-zein (C1)	24	−0.55**	46	−0.33*
15 kDa β-zein (C2)	24	n.s.	46	n.s.
C1 + C2 zein	24	−0.62***	46	−0.39**
27 kDa γ-zein (E)	24	n.s.	46	n.s.
16 kDa γ-zein (F)	24	n.s.	46	−0.31*

n.s. non-significant

*Significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$

helping explain previous reports showing no changes in ethanol yield with N fertilization rates [31].

Starch provides the feedstock (i.e., glucose) for ethanol fermentation. No correlation was observed between ethanol yield and starch concentration in exp 1, and a positive one was observed in exp. 2. Others have shown similar conflicting results between starch concentration and ethanol yield [6, 9, 32, 33]. Singh [8] suggested that because dry mill maize ethanol production process is not the direct chemical conversion of starch into ethanol but a complex process involving enzymatic starch hydrolysis and fermentation, it is impossible to predict ethanol yield based solely on kernel starch concentration. If calculated, our results show that higher starch concentrations were negatively correlated to the ethanol production efficiency per unit of available starch. This is in general agreement with previous studies [3, 11].

Proteins play an important role in determining maize ethanol yield. Zhan et al. [6] found a negative correlation between ethanol yield and protein concentrations in sorghum, and Lacerenza et al. [5] found similar results in barley and wheat. Our study confirmed this finding for maize, as evidenced by the negative correlations observed between protein and ethanol yields (Table 7). In exp. 1, the range of protein concentrations tested as a result of the experimental setup (i.e., year, kernel type, genotype, and N fertilization) was broader than the one observed in exp. 2. This is in line with the observation of a higher negative correlation between kernel protein concentration and ethanol yield observed in exp. 1.

Specific zeins are responsible for changes in kernel hardness [12, 17–19, 30]. The negative correlations found between ethanol yield and zeins (Table 7) evidence the involvement of these proteins in determining dry mill ethanol yield. Mature protein bodies have Z2 zeins accumulated on their peripheral zone and Z1 zeins as their filling [16]. These protein bodies constitute the endosperm protein matrix in which starch granules are embedded. Increased Z2 and Z1 zeins, leading to increased endosperm hardness, could decrease enzyme accessibility to starch granules or even reduce the level of gelatinized starch, diminishing the feedstock of fermentable sugars and, in consequence, yielding less ethanol. Ubach et al. [20] characterized low-fermentability maize as that holding high concentration of 15 kDa β-zein, 16 kDa γ-zein, and 19 kDa α-zein (one of the main components of Z1 [15]). Our results confirm the role of several of these zeins in determining maize fermentability. In the case of 15 kDa β-zein, which has two allelic variations, the concentration of C1, the most abundant β-zein in hard endosperm genotypes, was negatively correlated with ethanol yield. We hypothesize that this specific protein contributes to decrease the accessibility of amylases to the starch granules embedded within it, reducing the yield of glucose necessary for ethanol production. In fact, the use of proteases during dry grind ethanol production has increased the concentration of fermentable sugars [34] and ethanol yield [35].

Conclusions

Our results demonstrate that specific endosperm proteins affecting kernel hardness influence maize ethanol yield (L Mg^{-1}). This was tested by changes in kernel hardness through genotype selection, N fertilization, or environmental effects. Changes in ethanol yield were not correlated with kernel starch concentration consistently. Experiments showed reductions in ethanol yield related to endosperm kernel

density and kernel protein concentration, in particular the Z2 zeins 15 kDa β-zein and 16 kDa γ-zein.

Field ethanol yields (L ha^{-1}) were mostly related to changes in the crop field yield (Mg ha^{-1}) because differences in crop yield (Mg ha^{-1}) were much larger than differences in ethanol yields (L Mg^{-1}). Increasing N fertilization, for example, increased the field ethanol yield (L ha^{-1}); however, higher N fertilization rates led to lower ethanol yields on a mass basis (L Mg^{-1}).

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References

1. Wang H, Wang T, Johnson LA, Pometto AL III (2008) Effect of the corn breaking method on oil distribution between stillage phases of dry-grind corn ethanol production. J Agric Food Chem 56:9975–9980
2. Ramchandran D, Johnston DB, Tumbleson ME, Rausch KD, Singh V (2015) Seasonal variability in ethanol concentrations from a dry grind fermentation operation associated with incoming corn variability. Ind Crop Prod 67:155–160
3. Srichuwong S, Gutesa J, Blanco M, Duvick SA, Gardner C, Jane J (2010) Characterization of corn grains for dry-grind ethanol production. J ASTM Int 7:1–10
4. Naidu K, Singh V, Johnston DB, Rausch KD, Tumbleson ME (2007) Effects of ground corn particle size on ethanol yield and thin stillage soluble solids. Cereal Chem 84:6–9
5. Lacerenza JA, Martin JM, Talbert LE, Lanning SP, Giroux MJ (2008) Relationship of ethanol yield to agronomic and seed quality characteristics of small grains. Cereal Chem 85:322–328
6. Zhan X, Wang D, Tuinstra MR, Bean S, Seib PA, Sun XS (2003) Ethanol and lactic acid production as affected by sorghum genotype and location. Ind Crop Prod 18:245–255
7. Wu X, Zhao R, Bean SR, Seib PA, McLaren JS, Madl RL, Tuinstra M, Lenz MC, Wang D (2007) Factors impacting ethanol production from grain sorghum in the dry-grind process. Cereal Chem 84:130–136
8. Singh V (2012) Effect of corn quality on bioethanol production. Biocatal Agric Biotechnol 1:353–355
9. Singh V, Graeber JV (2005) Effect of corn hybrid variability and planting location on dry grind ethanol production. Trans ASAE 48:709–714
10. Dien BS, Wicklow DT, Singh V, Moreau RA, Winkler-Moser JK, Cotta MA (2012) Influence of *Stenocarpella maydis* infected corn on the composition of corn kernel and its conversion into ethanol. Cereal Chem 89:15–23
11. Gumienna M, Szwengiel A, Lasik M, Szambelan K, Majchrzycki D, Adamczyk J, Nowak J, Czarnecki Z (2016) Effect of corn grain variety on the bioethanol production efficiency. Fuel 164:386–392
12. Robutti JL, Borrás FS, Eyhérabide GH (1997) Zein compositions of mechanically separated coarse and fine portions of maize kernels. Cereal Chem 74:75–78
13. Pratt RC, Paulis JW, Miller K, Nelsen T, Bietz JA (1995) Association of zein classes with maize kernel hardness. Cereal Chem 72:162–167
14. Fox G, Manley M (2009) Hardness methods for testing maize kernels. J Agric Food Chem 57:5647–5657
15. Wilson CM (1991) Multiple zeins from maize endosperms characterized by reversed-phase high performance liquid chromatography. Plant Physiol 95:777–786
16. Lending DR, Larkins BA (1989) Changes in zein composition of protein bodies during maize endosperm development. Plant Cell 1:1011–1023
17. Eyhérabide GH, Robutti JL, Borrás FS (1996) Effect of near-infrared transmission-based selection on maize hardness and the composition of zeins. Cereal Chem 73:775–778
18. Robutti JL (1995) Maize kernel hardness estimation in breeding by near-infrared transmission analysis. Cereal Chem 72:632–636
19. Gerde JA, Tamagno S, Di Paola JC, Borrás L (2016) Genotype and nitrogen effects over maize kernel hardness and endosperm zein profiles. Crop Sci 56:1225–1233
20. Ubach MC, Jurado LA, Vinjamoori DV, Das P, Krohn B, Modiano SH (2007) Method of predicting a trait of interest. U.S. Patent Application WO 2007/118212 A1. Date issued: 18 October 2007
21. Tsai CY, Huber DM, Warren HL (1978) Relationship of the kernel sink for N to maize productivity. Crop Sci 18:399–404
22. Pearson CJ, Jacobs BC (1987) Yield components and nitrogen partitioning of maize in response to nitrogen before and after anthesis. Aust J Agric Res 38:1001–1009
23. Uhart SA, Andrade FH (1995) Nitrogen and carbon accumulation and remobilization during grain filling in maize under different source/sink ratios. Crop Sci 35:183–190
24. Tamagno S, Greco I, Almeida H, Martí Ribes F, Di Paola J, Borrás L (2016) Crop management options for maximizing maize kernel hardness. Agron J 108:1561–1570
25. Tsai CY, Huber DM, Glover DV, Warren HL (1984) Relationship of N deposition to grain yield and N response of three maize hybrids. Crop Sci 24:277–281
26. Ahmadi M, Wiebold WJ, Beuerlein JE, Kephart KD (1995) Protein quality of corn hybrids differing for endosperm characteristics and the effect of nitrogen fertilization. J Plant Nutr 18:1471–1481
27. Singletary GW, Doehlert DC, Wilson CM, Muhitch MJ, Below FE (1990) Response of enzymes and storage proteins of maize endosperm to nitrogen supply. Plant Physiol 94:858–864
28. Gambín BL, Borrás L (2005) Sorghum kernel weight: growth patterns from different positions within the panicle. Crop Sci 45:553–561
29. Borrás F, Seetharaman K, Yao N, Robutti JL, Percibaldi NM, Eyhérabide GH (2006) Relationship between popcorn composition and expansion volume and discrimination of corn types by using zein properties. Cereal Chem 83:86–92
30. Dombrink-Kurtzman MA, Bietz JA (1993) Zein composition in hard and soft endosperm of maize. Cereal Chem 70:105–108
31. Reicks G, Woodard HJ, Bly A (2009) Improving the fermentation characteristics of corn through agronomic and processing practices. Agron J 101:201–206
32. Yangcheng H, Jiang H, Blanco M, Jane J (2013) Characterization of normal and waxy corn starch for bioethanol production. J Agric Food Chem 61:379–386
33. Ai Y, Medic J, Jiang H, Wang D, Jane J (2011) Starch characterization and ethanol production of sorghum. J Agric Food Chem 59:7385–7392
34. Vidal BC, Rausch KD, Tumbleson ME, Singh V (2011) Corn endosperm fermentation using endogenous amino nitrogen generated by a fungal protease. Cereal Chem 88:117–123
35. Johnston DB, McAloon AJ (2014) Protease increases fermentation rate and ethanol yield in dry-grind ethanol production. Bioresour Technol 154:18–25

AUTHOR QUERIES

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Q1. The phone number has been distributed to all authors as contact information. Please check if correct.
Q2. Please check if the affiliation is presented correctly.
Q3. Please check if the sentence "However, and although these differences were as large...." is presented correctly.
Q4. Please check if section headings are assigned to appropriate levels.
Q5. Please check if edits to the sentence "Test weight was measured using a homogeneous...." retained its original meaning.
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