

Evaluation and comparison of protein composition and quality in half-sib families of *opaque-2* maize (*Zea mays* L.) from Argentina

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SUMMARY

The objective of this study was to characterize and compare the protein composition of half-sib families of *opaque-2* maize from Argentina through progeny testing. Grain hardness, protein fractions, amino acid content and *in vitro* protein digestibility (PD) of whole grain flour were determined. Non-opaque genotypes were used as control. Total protein content of *o2* genotypes correlated negatively with the grain hardness ($r=-.42$ in 2012 and $r=-0.32$ in 2013) and did not show significant differences in relation to non-opaque genotypes; however, *o2* genotypes showed significantly higher PD, lower zein proportion, and higher albumin, globulin and glutelin contents. The *o2* progenies showed significantly higher lysine content and higher levels of other essential amino acids, such as isoleucine and threonine compared to white corn. Lysine content, protein fraction contents and PD showed a higher contribution of genetic variance to the total variability, despite the high contribution of crop year variance obtained by total protein content. The differences shown by progeny protein profiles would enable the identification of *o2* genotypes with lower zein level and higher lysine content; this allows us to continue the selection and improvement to obtain open-pollination varieties enhanced in terms of protein quality.

Keywords: *opaque-2* maize, open-pollinated variety, lysine.

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RESUMEN

El objetivo fue caracterizar y comparar la composición proteica de familias de medios hermanos de maíz *opaco-2* de Argentina mediante prueba de progenie. Se determinó dureza del grano, contenido de fracciones proteicas,

aminoácidos y digestibilidad *in vitro* de proteína (PD) de la harina integral. Se usaron genotipos no-opacos como control. El contenido total de proteína de los genotipos *o2* correlacionó negativamente con la dureza del grano ($r=-42$ en 2012 y $r=-0.32$ en 2013) y no mostró diferencias significativas respecto a los genotipos no-opacos; sin embargo, los genotipos *o2* mostraron mayor PD, menor proporción de zeína y mayor de albúminas, globulinas y glutelinas. Las progenies *o2* mostraron mayor contenido de lisina, isoleucina y treonina en comparación con el maíz blanco. El contenido de lisina, el de fracciones proteicas y la PD mostraron una mayor contribución de la varianza genética, a pesar de la alta contribución de la varianza del año observada en el contenido total de proteínas. Las diferencias mostradas por los perfiles de proteínas de las progenies permitirán identificar genotipos *o2* con menor nivel de zeína y mayor contenido de lisina. Esto permitirá continuar con la selección y la mejora genética para obtener variedades con calidad proteica superior.

Palabras clave: maíz *opaco-2*, variedad de polinización libre, lisina.

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INTRODUCTION

Cereal foods have been the most important sources of dietary energy and nowadays maize is the primary and most important staple (Arendt and Emanuele, 2013). The mature corn kernel is composed of over 70 % of starch. Most of this starch (80 to 90 %) is found in the endosperm, which comprises about 80 % of the total kernel dry weight. Proteins constitute the second most abundant component in the corn kernel after starch, and generally range between 6 and 12 % of the grain dry weight (Serna-Saldivar, 2010). They are distributed mainly in the germ and endosperm and their characteristics differ significantly. The differences between the protein contents in maize can vary markedly according to the genotype, the grain physiological age and the environmental conditions during development and maturation of kernels in the field (Landry, Damerval, Azevedo and Sonia, 2005).

The maize endosperm is composed of an opaque flourey region rich in starch, which is located near the center of the kernel, and of another hard vitreous region rich in protein, located near the periphery

of the kernel (Hoseney, 1994). In normal maize, the endosperm proteins constitute 80 % of the total grain protein content (Arendt and Emanuele, 2013). In general, the albumins, globulins, prolamins and glutelins account for about 7, 5, 52 and 25 % of the whole kernel nitrogen, respectively. The zeins are the main prolamin, and they are deficient in lysine and tryptophan. They are arranged as insoluble aggregates (protein bodies) deposited in the rough endoplasmic reticulum lumen, forming a protein matrix. During the grain maturation, protein bodies are tightly packed between the starch granules in endosperm vitreous regions (Gayral *et al.*, 2016). The different classes of zeins can be distinguished by their different relative mobility on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), by their different solubility and by their different amino acid composition (Rodríguez-Nogales, Garcia and Marina, 2006).

The *opaque-2* maize has the recessive mutant gene (*o2*), which encodes a transcription factor (a gene regulator) of zein synthesis (Mertz, Bates and Nelson, 1964). The homozygous *o2* mutant causes a decrease of the α -zein production, resulting in a redistribution of nitrogen in the endosperm to

non-zein fractions, as well as the accumulation of free amino acids. Since the non-zein proteins contain higher lysine and tryptophan amount, the effective content of these amino acids of the grain is elevated (Gibbon and Larkins, 2005; Kriz, 2009). The zein synthesis reduction could be related to the low ability of protein bodies to store them (Landry *et al.*, 2005), but it could also result from the cessation of protein synthesis as was suggested by Habben, Kirleis and Larkins (1993). The response of the endosperm containing *o2* mutation have been estimated by quantifying their zein or lysine content (Landry *et al.*, 2005).

However, the *o2* grains have soft endosperm, and therefore susceptible to pests and fungal diseases, mainly during storage (Vivek, Krivanek, Palacios-Rojas, Twumasi-Afriyie and Diallo, 2008). The progress in the improvement of *o2* genotypes with higher lysine contents and endosperm hardness, resulted from the soft grains phenotype suppression by endosperm hardness modifier genes, which convert the soft endosperm to a hard/vitreous endosperm, resulting in the QPM (quality protein maize) varieties (Prasanna, Vasal, Kassahun and Singh, 2001; Sofi, Wani, Rather and Wani, 2009). These modifiers along with the *o2* mutant allele can be selected using a method of selection whereby light is projected through the vitreous grains or blocked by the opaque grains respectively (Krivanek, Groote, Gunaratna, Diallo and Friesen, 2007; Vivek *et al.*, 2008). Therefore, in breeding programmes, the selection of genotypes with higher endosperm hardness is essential to reduce the soft phenotype and improve the grain physical traits, resulting in major agronomical acceptance (Krivanek *et al.*, 2007; Gayral *et al.*, 2016).

Multiple genes have been identified as responsible for controlling amino acid content; they affect the relative levels of lysine and tryptophan in the grain endosperm (Wang, Woo, Kim, and Larkins, 2001). Thus, it has become apparent that the simple genetic nature of *opaque-2* maize has transformed into a classic polygenic trait and must be manipulated as such in breeding programs. If lysine or tryptophan levels are not continuously measured during the breeding process, the additional gains in protein quality may be lost even though the *o2o2* genotype is maintained (Krivanek *et al.*, 2007).

Argentina has a great diversity of maize germplasm due to its wide variation in latitude and altitude. The largest grown area in the country is covered by hybrid varieties due to the high heterosis of this species, resulting in a positive rate in the amount of commercial seed obtained per unit

area (Rossi, 2007). However, the annual purchase of hybrid seeds represents a high fixed cost, therefore, only producers with substantial economic resources and technologies may remain in the production system. On the other hand, in areas where small farmers do not have economic access to hybrid seeds, the open-pollinated varieties (OPV) prove to be a good choice since producers can keep their own seeds for the following planting season. In addition, the OPV genetic variability allows these varieties to adapt easily to different weather and soil conditions (Kutka, 2011).

Many researchers have suggested that modified ear-to-row method, based on the among-and-within selection of half-sib families, is an effective method of recurrent selection in maize (Acquaah, 2012). In fact, the half-sib selection is based on general combining ability since the entire population serves as a tester for pollination of female plants. The success of the selection is assured by the presence of superior genotypes, which in turn depends on sample size and heritability of selected traits. In maize, this method has been used efficiently for the genetic improvement of high heritability agronomic traits and in the protein and oil content in the kernel (Acquaah, 2012).

Since the discovery of *o2* mutation, a lot of studies have been conducted to develop varieties that improve consumers' nutrition, especially in developing countries (Vivek *et al.*, 2008; Gunaratna, Groote, Nestel, Pixley and McCabe, 2010). Currently, *opaque-2* and QPM varieties are being disseminated particularly in America and Africa (Krivanek *et al.*, 2007; Gunaratna *et al.*, 2010). In the American continent, the development of cultivars is very widespread in Mexico, Central America and Southern United States and the consumption of *opaque-2* and QPM varieties is spread mainly in the form of *tortillas* and nixtamalized products (Rojas-Molina *et al.*, 2008). Some studies have focused on the genetic improvement of this germplasm in commercial maize areas from Argentina (Magoja and Nivio, 1982; Magoja, Nivio and Streitenberger, 1984; Corcuera, Salmoral, Kandus, Ferrero and Salerno, 2013; Corcuera, Salmoral, Pennisi, Kandus and Salerno, 2016). The introduction, adaptation, recombination and selection of genotypes would be useful to provide important genetic resources to local producers; at the same time, selected genotypes represent sources of raw material for food production. Therefore, the objective of this study was to characterize and compare the protein composition of half-sib families of *opaque-2* maize grown in Argentina in two crop years.

MATERIALS AND METHODS

Genetic material

An *opaque-2* maize germplasm provided by International Maize and Wheat Improvement Center (CIMMYT), México, was used as starting material (OP). An adaptation seeding was performed (planting date December 2011) at the experimental field of Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina (31°28' 49.42" S, 64°00' 36.04" W). Twelve main cobs of different plants with healthy features were selected (half-sib families) from the original population (OP) and individually harvested (June 2012). After harvest, the cobs were individually threshed, and the opaque grains of each cob were selected using a light table according to Vivek *et al.* (2008). Approximately 90 opaque kernels of each cob were stored at 4 °C until planting; the rest were milled in the laboratory to obtain the whole grain flour. In December 2012, the seeds of each cob were planted in individual rows under a randomized complete block design with three replications in order to obtain their individual progenies. The experimental plots were composed of 12 rows (row-family) of 5 m long, with a spacing of 0.70 m between rows. In June 2013, the cobs of the best plants within each progeny were harvested (grain moisture at harvest in both years was around 18 %) and the opaque grains were selected (Vivek *et al.*, 2008). Grains were then stored and milled under

the same conditions as those of the 2012 harvest. The field trials in both crop years (December 2011 - June 2012 and December 2012 - June 2013) were conducted in the same experimental field (same location) but in different plots uncultivated for a period of three years. The assays were carried out both years under dry conditions without nitrogen fertilization, in order to compare both crops under equal experimental conditions. Table 1 shows the average monthly temperature and precipitation for both crop years.

The opaque grains selected from genotypes harvested in both crop years (twelve half-sib families grown in 2012, and their individual progenies obtained in 2013) were milled in a cyclonic mill (Cyclotec CT193, Foss, Suzhou) without previous drying. Whole grain flour was obtained (moisture around 12 to 15 % and particle size range less than 500 µm) and stored until chemical analysis. The original population (OP) used as starting material and two non-opaque genotypes (a white corn synthetic variety, WC, and an open-pollinated popcorn variety, PC) were used as controls for comparison.

Physico-chemical composition

The moisture, protein (N x 6.25), lipid and ash contents of whole grain flour were determined according to American Association of Cereal Chemists International (AACC International)

Table 1. Average monthly temperatures and total precipitation of crop years

Crop year month	Temperature			Total precipitation (mm)
	Minimum (°C)	Maximum (°C)	Average (°C)	
1)2012				
January	16.2e	32.7e	24.4f	142.2
February	17.9e	29.6d	23.8f	166
March	13.6d	28.4d	21.0e	54
April	11.6d	24.4c	18.0d	22.2
May	9.4c	23.1b	16.2c	10
June	3.8a	19.6a	11.7a	0.8
Average	12.1	26.3	19.2	
2013				
January	16.9e	31.7e	24.3f	95.6
February	15.5e	28.2d	21.8e	90.6
March	12.6d	25.8c	19.2d	44.4
April	10.2c	26.2d	18.1d	47.4
May	7.2b	21.9b	14.5b	33
June	4.5a	20.2a	12.4a	2
Average	11.2	25.6	18.4	

Values followed by different letters in the same column are significantly different ($P < 0.05$).

1) Grain-filling occurred during April and May in both crop years.

Approved Methods 44-19.01, 46-13.01, 30-25.01 and 08-01.01, respectively (AACC International, 2010). All determinations were measured at least in duplicate and expressed as g 100 g⁻¹ of flour on dry basis (db). Resistant starch (RS) content was determined by enzymatic Resistant Starch assay procedure utilizing commercial K-RSTAR® kit (Megazyme International, Ltd., Bray, Ireland), as described by Mansilla, Nazar and Pérez, 2017. Before such determination, the whole grain flour (100 mg) of each genotype was defatted, suspended in 1.6 mL of distilled water, and cooked in a boiling water bath for 12.30 min in order to allow starch gelatinization by simulating a cooking process. Then, samples were incubated with pancreatic α -amylase and amyloglucosidase (AMG) in a shaking water bath for 16 h at 37 °C to solubilize and hydrolyze non-resistant starch (NRS) to D-glucose by the combined action of the two enzymes. Subsequently, the NRS was separated by centrifugation; the RS-containing pellet was purified with ethanol, solubilized with 2M KOH, and hydrolyzed to glucose with AMG. Reagent glucose oxidase/oxidase was used to quantify D-glucose by spectrophotometer at 510 nm. RS plus NRS (digestible starch) was considered total starch content and was expressed as g 100 g⁻¹ of flour (db). All determinations were measured at least in duplicate.

Flotation index (FI) was determined as an indirect measure of grain hardness (Narváez-González *et al.*, 2006). One hundred grains were placed in a beaker containing 500 mL of 67 % saccharose solution (density of 1.25 g mL⁻¹). They were stirred and left standing for 1 min. The number of floating grains indicated the flotation index. Hardness classification was based on the scale proposed by Salinas, Bustos and Gomez (1992): FI values between 0 and 12 % are very hard grains, 13 to 37 % are hard grains, 38 to 62 % are intermediate grains, 63 to 87 % are soft and more than 87 % are very soft. All determinations were measured at least in duplicate.

Extraction and quantification of protein fractions

A sequential extraction procedure of maize protein was carried out following the method described by Malumba, Vanderghem, Deroanne and Béra (2008) with modifications. The albumin fraction (AL) was twice extracted by stirring defatted flour with distilled water at 4 °C for 1 h and centrifuged for 15 min at 18000 g (Thermo Scientific, Sorvall ST40R). The meal residues

from water-soluble extraction were stirred at room temperature with 0.5 M sodium hydroxide (NaCl) and then centrifuged at 18000 g for 15 min to remove globulin fraction (GL). Zein fraction (Z) was solubilized with a 70 % ethanol + 0.5 % sodium acetate solution, and the second zein fraction (Z2) with 70 % ethanol + 0.5 % sodium acetate + 0.6 % Dithiothreitol (DTT). Glutelin G2 fraction was extracted with borate buffer pH 10 + 0.6 % DTT and glutelin G3 fraction with borate buffer pH 10 + 0.6 % DTT + 0.5 % of sodium dodecyl sulfate (SDS). The protein content of each fraction (N x 6.25) was determined by the micro-Kjeldahl method modified to boric acid (AACC International, 2010). All determinations were measured at least in duplicate and the extracts were stored at -18 °C. The results were expressed as protein percentage of each fraction from the flour (g protein fraction 100 g⁻¹ of flour) (db).

Electrophoresis

Electrophoresis was performed in polyacrylamide SDS gels (SDS-PAGE). The protein extracts were precipitated with acetone (1:3) and reduced by heating at 95 °C for 5 min in a sample buffer containing 0.063 M Tris/HCl pH 6.8, 2 % of SDS p/v, 10 % of glycerol v/v, 5 % of β - mercaptoethanol and 0.05 % of bromophenol blue p/v (Duodu *et al.*, 2002). The separating gel concentrations were 12 % acrylamide for albumin, globulin and glutelin fractions, and 15 % acrylamide for the zein fraction. Runs were stopped when the running front reached the end of the gel, and then stained using Coomassie blue. A Bio-Rad SDS-PAGE Standards, Broad Range (200 to 6.5 kDa) marker was used to determine the molecular weights of the polypeptide bands of the extracts.

Amino acid content

Samples of whole grain flour (1 g, db) were hydrolyzed with 50 mL of hydrochloric acid (HCl) 6M and were heated in reflux in a heating mantle for 24 h, according to AOAC International (1999) Method 994.13. The derivatization was performed according to the method described by Alaiz, Navarro, Girón and Vioque (1992). Amino acids were determined with an HPLC Perkin Elmer, Serie 200 equipment with an UV-visible detector (280 nm), using a Zorbax Eclipse Plus C18 (4.6 x 150 mm) column, and a particle size 5 μ m (Agilent Technologies). The amino acid content was expressed in g 100 g⁻¹ of protein (db), and all determinations were measured in quadruplicate.

***In vitro* protein digestibility**

In vitro protein digestibility was determined according to the procedure described by Hamaker, Kirleis, Butler, Axtell and Mertz (1987), with modifications. Samples of whole grain flour (200 mg, db) of each genotype were suspended in 2 mL of distilled water and cooked in a boiling water bath for 10 min, in order to simulate a cooking process (Duodu *et al.*, 2002). Then, samples were cooled at room temperature and centrifuged during 10 min at 4000 g. The residues were suspended in phosphate buffer 0.1 M pH 2 with pepsin (Sigma P-7000-25G, activity 683 U mg⁻¹ of protein) and incubated in a shaking water bath at 37 °C. Pepsin digestion was stopped at 2 h with the addition of 2 mL of 2 M NaOH. An aliquot (1.5 mL) was centrifuged at 14000 g for 10 min at 25 °C and the solubilized protein in the supernatant was determined by Lowry method (Waterborg, 2002). The absorbances were measured in a spectrophotometer (UV-visible JascoV-730, Jasco Corporation, Japan) at 550 nm and a calibration pattern with bovine serum albumin (Sigma A2153-10G, lyophilized powder >96 %) was used as standard, at concentrations from 0.01 to 2 mg mL⁻¹. The percentage of digested protein with respect to the total protein content was considered as *in vitro* digestibility. All determinations were measured at least in duplicate.

Statistical analysis

Statistical analyses were performed using Infostat/Professional statistical software (Di Rienzo *et al.*, 2018). The data were examined by ANOVA and Di Rienzo, Guzmán and Casanoves's multiple comparison test (DGC) (Di Rienzo, Guzmán and Casanoves, 2002) was applied. The differences between genotypes (average of each genotype from the two harvests, 2012 and 2013) and crop years (average of all genotypes from each year, 2012 or 2013) were analyzed. In addition, the individual progenies were compared with the original population used as starting material and with two non-opaque genotypes (white corn and popcorn) used as controls.

The variance components were estimated by Linear Mixed Models (MLM) through Restricted Maximum-Likelihood estimator (REML). The genotype factor (progenies) was taken nested within the environment factor (crop year), both as random effects, and the residual variance was considered as the interaction between both factors (Di Rienzo, Macchiavelli and Casanoves, 2017). In this way, it was possible to assess the relative magnitude of the

genetic variance (given by the genetic differences between genotypes) of environment (given by crop year) and of the genotype x year interaction (given by residual variance), determined as percentage of the contribution to the total variance. Non-opaque controls were not included in the analysis. The relationships between parameters were determined by Pearson's correlation test (significant level at $P < 0.05$).

RESULTS AND DISCUSSION

Grain composition

The *opaque-2* genotypes showed significant differences between total starch and protein content, but the average values did not show significant differences when compared with white corn and popcorn (Table 2). These results have already been discussed in a previous research focused on starch properties of these *opaque-2* genotypes (Mansilla *et al.*, 2017). On the other hand, Ortiz-Martinez, Otero-Pappatheodorou, Serna-Saldívar and García-Lara (2017) obtained significantly higher protein content in QPM corn (13.5 %) in relation to normal corn (9.25 %); these differences were attributed to the contribution of the germ protein. Cázares-Sánchez, Chávez-Servía, Salinas-Moreno, Castillo-González and Ramírez-Vallejo (2015) reported a similar protein range when they analyzed 41 populations of Mexican native corns (9.35 - 12.50 %) with different types of endosperm. The *opaque-2* progenies showed higher FI than non-opaque controls (Table 2), indicating that *opaque-2* maize had very soft grains compared with intermediate and very hard grains of white corn and popcorn, respectively. *Opaque-2* maize contains a higher proportion of floury endosperm, with a dull appearance due to the loose packing of starch granules (Gibbon and Larkins, 2005). Similar results were demonstrated by Narváez-González *et al.* (2006) in grain hardness of maize races from México. Previous reports (Joshi, Lodha and Mehta, 1980) stated that *opaque-2* maize endosperm has a lower starch amount than normal maize, as reported by Ortiz-Martinez *et al.* (2017) who obtained a significantly lower starch content in a QPM variety (64.14 %) in relation to normal corn (73.26 %). However, Pereira *et al.* (2008) demonstrated that the highest total starch levels were both in genotypes with hard and soft endosperm, so they rejected an association between starch content and endosperm hardness, in agreement with the present study.

Table 2. Grain hardness, flour chemical composition and *in vitro* protein digestibility of maize genotypes

1) Genotype	Flotation Index (%)	Centesimal composition (g 100 ⁻¹ g of flour)				Protein composition (g 100 ⁻¹ g of flour)			PD (%)
		Starch	Lipid	Ash	Protein	Zeins			
						2) Z+Z2	AL+GB	G2+G3	
P 3	91.8±3.7c	61.9±3.4a	10.9±0.9d	1.9±0.2b	11.6±0.5d	3.7±0.6f	3.4±0.2b	2.8±0.4a	74.9±1.7b
P 5	86.5±2.8b	65.5±1.3b	7.7±1.3b	1.7±0.1a	11.3±0.4c	2.2±0.1c	3.3±0.2b	3.2±0.1b	59.1±5.8a
P 10	97.8±4.2d	63.6±1.1b	8.5±0.56c	1.8±0.2a	11.0±0.2c	2.2±0.2c	2.9±0.3a	3.8±0.7d	68.5±8.6b
P 13	84.8±2.2b	66.3±2.3b	7.9±2.6b	1.9±0.1b	10.1±0.7a	2.2±0.4c	4.2±0.6d	4.4±0.2e	72.3±2.7b
P 15	96.0±2.9d	63.3±3.1b	8.7±0.6c	2.0±0.0c	10.1±0.9a	1.7±0.1a	3.6±0.5c	3.2±0.1b	67.7±8.9b
P 18	97.0±5.7d	64.5±2.8b	8.8±2.3c	1.9±0.1b	9.8±1.7a	2.1±0.3b	3.8±0.2c	3.5±0.2c	89.4±3.0c
P 19	94.5±7.1c	60.9±1.0a	8.1±0.4b	1.9±0.1b	11.1±0.9c	2.4±0.5c	4.4±0.7e	3.4±0.3c	70.4±7.3b
P 20	88.5±3.7b	65.2±1.3b	8.6±0.6c	1.8±0.3a	10.1±0.6a	2.3±0.1c	3.4±0.1b	2.9±0.1a	89.1±8.0c
P 22	98.5±5.5d	65.7±2.1b	9.1±0.8c	2.1±0.1d	10.4±0.6b	1.6±0.2a	4.1±0.4d	3.5±0.2c	91.1±6.4c
P 25	92.0±1.9c	63.5±1.3b	6.7±0.8a	2.0±0.1c	11.9±0.7d	2.2±0.1c	4.1±0.4d	3.5±0.4c	84.6±2.0c
P 26	86.0±4.9b	61.8±2.2a	7.7±1.5b	1.9±0.2b	10.5±0.8b	2.7±0.2e	3.6±0.4c	3.4±0.3c	61.9±7.6a
P 28	81.3±1.2a	66.0±4.5b	8.0±1.4b	2.1±0.2d	12.3±0.8e	2.5±0.4d	4.1±0.1d	3.2±0.2b	80.1±6.7c
OP	98.7±8.1d	61.2±1.3a	8.6±0.1c	2.1±0.1d	10.6±0.3b	2.1±0.1b	3.4±0.0b	3.3±0.1c	85.3±7.0c
3) Year									
2012	94.6±9.1b	65.0±3.9b	8.2±1.6a	1.9±0.2a	10.3±0.9a	2.2±0.7a	3.9±0.6b	3.4±0.5a	74.1±9.7a
2013	89.2±8.50a	62.6±2.7a	8.6±1.4b	2.0±0.3b	11.4±0.8b	2.4±0.4b	3.6±0.6a	3.4±0.4a	78.9±8.9b
4) Comparison									
Opaque	91.9±7.2c	63.8±3.5a	8.4±1.5b	1.9±0.2b	10.8±1.1a	2.3±0.6a	3.7±0.6b	3.4±0.5b	76.5±11.8b
WC	62.0±2.1b	66.3±1.3a	6.7±0.9a	1.7±0.1a	11.2±0.6a	4.9±0.0b	2.0±0.1a	2.4±0.1a	70.0±1.8a
PC	12.0±3.2a	66.2±0.8a	7.8±0.4a	1.5±0.0a	10.3±0.4a	4.7±0.1b	2.0±0.0a	2.9±0.0a	67.6±1.5a

Values followed by different letters in the same column are significantly different ($P < 0.05$).

¹⁾ Values of each genotype (P followed by a number) and Original Population (OP) correspond to the average between both crop years (2012 and 2013). Parameters expressed and compared on dry basis.

²⁾ Z+Z2 = zeins; AL+GB = albumins + globulins; G2+G3 = glutelins; PD = *in vitro* protein digestibility.

³⁾ Comparison of annual averages of all *opaque-2* half-sib families harvested in 2012 and their individual progenies grown in 2013. The data shown for 2012 correspond to the average of the twelve *opaque-2* half-sib families harvested in that year. Data shown for 2013 correspond to the average of their twelve individual progenies grown in 2013, in order to compare annual average variation on analyzed genotypes.

⁴⁾ Comparison of the average between all *opaque-2* genotypes (Opaque) in both crop years with white corn (WC) and popcorn (PC) used as controls.

Significant differences were obtained in lipid and ash contents of *opaque-2* genotypes, whose average values were higher than those obtained in white corn and popcorn used as controls (Table 2), as described in a previous work (Mansilla *et al.*, 2017). Lower lipid and ash contents were reported by Corcuera *et al.* (2013) and Mendoza-Elos *et al.* (2006) in experimental hybrids. On the other hand, Cázares-Sánchez *et al.* (2015) reported lower lipid levels in 41 native populations of Mexican maize. No significant correlations were obtained between lipids with protein, starch and ash contents in any crop year, in agreement with other studies (Cázares-Sánchez *et al.*, 2015).

An annual increase in protein content and a decrease in starch content were observed in *opaque-2* genotypes (Table 2). The lower amount

of starch in corn endosperm results in an increase in protein content (Serna-Saldivar, 2010), which was coincident with a negative correlation between protein and starch in both crop years ($r = -0.43$ in 2012 and $r = -0.51$ in 2013; $P < 0.05$) within *o2* genotypes. Several research studies proved that prevailing high temperatures during grain filling cause an increase in protein content and a decrease in starch, inducing changes in physicochemical properties (Beckles and Thitisaksakul, 2014). On the other hand, other works showed an increase in protein contents of experimental and commercial hybrids grown under drought conditions in relation to those cultivated under well-watered environments (Scott, Edwards, Bell, Schussler and Smith, 2006). The environmental effect is greater in the beginning of grain filling due to the interference of physiological

and biochemical cellular processes, and to an alteration of enzymatic activity (Thitisaksakul, Jiménez, Arias and Beckles, 2012). In the present study, there was a significant increase in the average maximum temperature of April 2013 in relation to 2012, month during which the grain filling took place (Table 1). However, the rainfall recorded in the same period was higher in 2013, which does not suggest water stress conditions during grain development. This would indicate that mainly high temperatures could have influenced the biosynthesis of grain components and could have translated into the annual variations observed in the progenies in 2013 (Table 2).

On the other hand, the protein of maize grain can be modified by soil nitrogen level, as demonstrated in other studies. The variations in nitrogen levels have a direct relationship with the protein/starch ratio of kernels (Ngaboyisonga, Njoroge, Kirubi and Githiri, 2008). In this study, nitrogen fertilization was not performed in any crop cycle, and experimental conditions in both years were similar.

Protein composition

Maize protein fractions were quantified and grouped as zeins and non-zeins (albumins + globulins and glutelins). Significant differences were obtained between *opaque-2* genotypes (Table 2). Landry, Delhaye and Damerval (2004) reported similar glutelin (3.48 %), higher zein (6.85 %) and lower albumin + globulin (0.65 %) contents in inbred lines of flourey maize in relation to those shown by *o2* genotypes of this study (Table 2). The decrease in zein synthesis of *o2* mutant genotypes could be related to a low storage capacity of endosperm protein bodies. This promotes the non-zein synthesis as a compensatory effect (Landry *et al.*, 2005), resulting in higher albumin, globulin and glutelin contents in the mature kernel. Ortiz-Martinez *et al.* (2017) reported albumin and globulin contents in QPM varieties similar to those obtained in *o2* genotypes of this study (Table 2). The synthesis and accumulation of albumins and globulins decline rapidly during grain maturation of normal endosperm genotypes in relation to *o2* and QPM genotypes. However, the final albumin + globulin contents in mature grain of these genotypes are twice as much as in normal endosperm corns (Wall and Bietz, 1987; Ortiz-Martinez *et al.*, 2017).

The P3 progeny showed significantly higher zein content and lower glutelin content than the rest of the genotypes (Table 2). This sample also had one of the highest total protein contents (Table 2). The zein synthesis shows a linear relationship based

on total protein content regardless of environment conditions (Landry *et al.*, 2005). Conversely, the P15 and P22 progenies showed a significantly lower zein content. In addition, progeny P22 was located between the samples with higher albumin + globulin and glutelin contents than the rest of the genotypes and in relation to the original population (OP) (Table 2). This indicates differences in protein composition with the starting genetic material.

When the protein fraction distribution was analyzed in relation to the total protein content, the average value of zein proportion within *opaque-2* genotypes (21.09 %) was lower than that of albumins + globulins (34.64 %) and glutelins (31.83 %), and significantly lower than the zein proportion of popcorn (45.71 %) and white corn (43.46 %). In the same way, the proportion of albumins + globulins and glutelins of white corn (18 % and 21 %, respectively) and popcorn (19.55 % and 28.27 %, respectively) were significantly lower than *o2* genotypes. Similar results were reported by Yau, Bockholt, Smith, Rooney and Waniska (1999) in MO17 experimental inbred line, where zein proportions in their *o2* and QPM homologous versions were significantly lower than their normal counterparts and they did not show differences in total protein content. The *o2* mutation in homozygous state modifies the corn protein quality but does not change the total protein content of the kernel (Gibbon and Larkins, 2005; Vivek *et al.*, 2008). Malumba *et al.* (2008) reported similar results in flint corn to those obtained in white corn and popcorn in the present study.

The zein fraction showed a negative correlation with glutelin ($r=-0.71$ in 2012 and $r=-0.41$ in 2013; $P<0.05$) and albumin + globulin fractions ($r=-0.69$ in 2012 and $r=-0.74$ in 2013; $P<0.05$) within *opaque-2* genotypes. In addition, the albumins correlated positively with globulins ($r=0.53$ in 2012 and $r=0.47$ in 2013; $P<0.05$). These results are coincident with other research studies in which the protein composition variations of *o2* mutants in relation to normal genotypes were attributed mainly to a decrease in zein fractions (Gibbon and Larkins, 2005; Ortiz-Martinez *et al.*, 2017). On the other hand, FI correlated negatively with protein ($r=-0.42$ in 2012 and $r=-0.32$ in 2013; $P<0.05$) and zein ($r=-0.38$ in 2012 and $r=-0.35$ in 2013, $P<0.05$) contents within *o2* progenies, in agreement with other studies (Narváez-González *et al.*, 2006). The zeins are closely related to endosperm hardness because they allow the association between starch granules with the protein matrix (Dombrink-Kurtzman and Bietz, 1993). Despite the *opaque-2* genotypes have very soft grains, P13 and P28 showed lower FI and high albumin + globulin and glutelin fractions

(Table 2), being essential to identifying progenies with high non-zein fractions and better physical features such as grain hardness.

When the variations between crop years in *opaque-2* genotypes were compared, the average annual increase in total protein content was accompanied by an increase in zein fraction content and a decrease in FI (Table 2). Concomitantly, a positive correlation between protein and zeins was observed in both crop years ($r=0.45$ in 2012 and $r=0.37$ in 2013; $P<0.05$). The albumin + globulin fractions showed a decrease from one year to another (Table 2). However, the glutelin fraction did not show a significant annual variation despite the positive correlation obtained with albumins + globulins ($r=0.42$ in 2012 and $r=0.54$ in 2013; $P<0.05$). These relationships were similar to those reported by Yau *et al.* (1999).

In vitro protein digestibility

The *opaque-2* genotypes showed significant differences in protein digestibility in cooked flour, whose average value was higher than white corn and popcorn (Table 2). Duodu *et al.* (2002) indicated that a tighter packaging degree of starch granules in the protein matrix of vitreous endosperm may affect the accessibility of digestive enzymes to flour particles. In this study, the higher vitreous endosperm proportion of popcorn and white corn would probably explain the lower protein digestibility obtained with respect to *o2* genotypes. The P22 progeny showed the highest value, although it did not differ significantly from the P18, P20, P25, P28 and OP samples (Table 2). Lower *in vitro* protein digestibility values were obtained by Duodu *et al.* (2002) in cooked and uncooked white

corn flours and whole grain corn flours subjected to pepsin digestion (from 62 to 68 %).

Some previous studies reported significant reductions in protein digestibility from sorghum whole grain flours after cooking. However, corn flours showed higher digestibility compared to sorghum (Hamaker, Kirleis, Mertz and Axtell, 1986; Duodu *et al.*, 2002). In sorghum and maize, the cooking process leads to the formation of oligomeric proteins linked by disulfide bridges that surround monomeric prolamines. These protein structures depend on prolamins composition and conformation of each cereal and they are weaker in corn than in sorghum (Hamaker *et al.*, 1986). This may result in less resistance to enzymatic action, which probably causes higher corn protein digestibility (Duodu *et al.*, 2002). In this study, the endosperm zein content of maize genotypes probably represented one of the main structural factors of grains that influenced the protein digestibility after cooking, as was observed in the negative correlation between zeins and protein digestibility obtained in the 2013 progenies ($r=-0.40$; $P<0.05$). In addition, a joint increase from one crop year to another was observed in protein digestibility and in the zein content of *opaque-2* genotypes (Table 2), which could be related to the increase in total protein content in 2013, indicating a higher amount of substrate for protein digestion.

Electrophoresis of protein fractions

An electrophoresis of each extract was performed in polyacrylamide gels (SDS-PAGE). Within *opaque-2* genotypes, zein fraction showed a similar band pattern in both crop years and molecular weights ranged from 6.5 to 45 kDa (Figure 1). Albumin (from 21 to 97 kDa), globulin

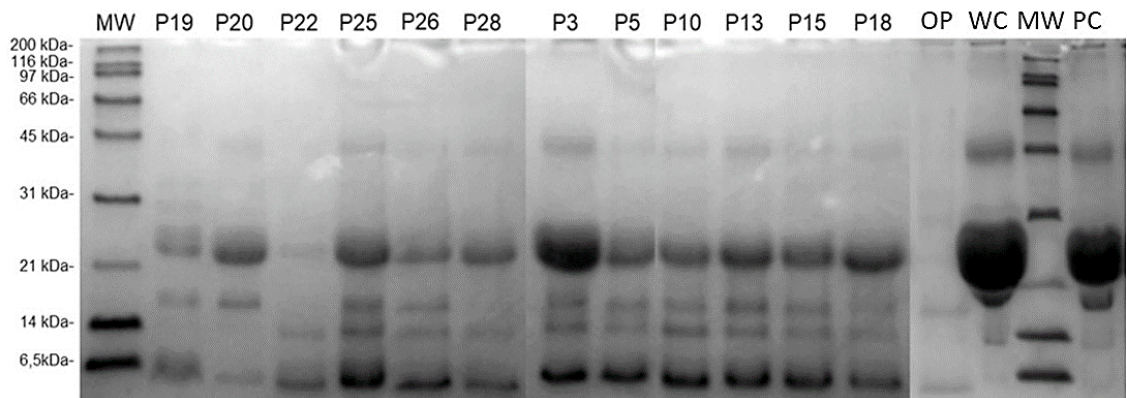


Figure 1. SDS electrophoretic comparison of zeins from whole endosperm of the maize genotypes. The lanes represent the half-sib families of *opaque-2* maize selected in 2012 (P followed by a number), the original population (OP), the white corn (WC) and popcorn (PC) used as controls. MW, molecular weight marker.

(from 14 to 66 kDa) and glutelin (from 21 to 66 kDa) patterns did not show great differences in the intensity of the bands between genotypes (data not shown). Landry *et al.* (2004) reported a range from 14 to 22 kDa in zein protein extracts of vitreous and floury corn endosperm. The P3 sample showed the highest intensity of the bands between 21-31 kDa and 45 kDa in 2012 with respect to the other *opaque-2* samples (Figure 1), which was consistent with the highest zein content shown by that sample in both crop years (Table 2). Conversely, the lowest zein content of P22 in both crop years (Table 2) was coincident with the lower band pattern intensity shown by this sample in 2012, in relation to the other *o2* genotypes (Figure 1).

When comparing electrophoretic patterns of non-opaque controls with *opaque-2* genotypes, a marked increase was observed in the intensity of 21-31 kDa and 45 kDa molecular mass bands in white corn and popcorn (Figure 1). This was reflected on the significantly higher zein content shown by these samples with respect to *o2* genotypes (Table 2). Some studies showed that the hard endosperm region of the corn kernel has higher α -zein (22 and 19 kDa) and γ -zein (27 and 16 kDa) contents and that the 27 kDa γ -zein determines the vitreous endosperm formation (Dombrink-Kurtzman and Bietz, 1993). However, in this electrophoretic study, a single broad band comprised between 22 and 30 kDa was observed and it could not be seen as two distinct bands due to the high zein concentration and the similar molecular mass of the zein bands. Malumba *et al.* (2008) reported a similar zein electrophoretic pattern in flint corn genotype, where the molecular mass bands between 20 and 30 kDa

showed higher intensity. On the other hand, Pereira *et al.* (2008) demonstrated a decrease in 22 and 19 kDa zein band intensity of *o2* mutant genotype with respect to QPM and hard endosperm samples. However, other studies indicated that there is no clear relationship between the appearance of 27 kDa γ -zein and vitreous endosperm in maize (Landry *et al.*, 2004; Gibbon and Larkins, 2005).

Amino acid analysis

Based on protein fraction contents and their electrophoretic patterns, the amino acid profile was determined: in *opaque-2* genotypes that showed the highest zein content in both crop years (P3) (Table 2), in those that presented the lowest zein levels (P15 and P22) (Table 2), in the original population (OP), and in the white corn (WC) used as non-opaque control for comparison. Significant differences were obtained in the amino acid profiles among analyzed genotypes and the lysine contents of *o2* progenies were significantly higher than white corn (Table 3). Considerably lower lysine contents than *opaque-2* genotypes and similar to the white corn used as control in the present study were reported in native populations of Mexican corns by Cázares-Sánchez *et al.* (2015) and Vera-Guzmán, Chávez-Servía and Carrillo-Rodríguez (2012) (0.27 - 0.39 and 0.4 - 0.6 g 100 g⁻¹ of protein, respectively). However, other studies reported higher lysine contents in hybrids, and *o2* and QPM lines (Wall and Paulis, 1978; Magoja *et al.*, 1984; Sofi *et al.*, 2009; Corcuera *et al.*, 2016).

Table 3. Amino acid content (g 100⁻¹ g of protein) of maize genotypes

	¹⁾ P 3	P 15	P 22	OP	Opaque average	WC
Aspartic Acid	3.96±0.20c	6.35±0.58e	5.68±0.80d	3.02±0.34b	4.92±1.42	1.91±0.01a
Glutamic Acid	6.41±1.69c	6.45±1.31d	2.71±0.36a	4.26±0.87b	5.06±1.37	3.94±0.03b
Glycine	0.52±0.12b	1.42±0.48c	0.83±0.08b	0.57±0.12b	0.86±0.39	0.09±0.01a
Threonine	0.87±0.02a	1.85±0.26b	2.20±0.20b	0.70±0.13a	1.50±0.86	0.31±0.00a
Arginine	5.34±0.03a	5.03±1.23a	7.12±1.83b	4.96±0.64a	5.62±2.03	2.34±0.00a
Alanine	6.97±1.29c	5.48±0.62b	4.19±0.89a	5.47±0.20b	5.43±1.46	6.02±0.03b
Proline	2.93±0.19a	2.63±0.32a	6.92±1.05b	4.77±1.42b	4.40±2.13	5.10±0.28b
Tyrosine	2.30±0.94c	1.80±0.22b	1.45±0.56b	1.05±0.56a	1.58±0.73	1.70±0.15b
Isoleucine	2.58±0.44a	3.18±0.59a	2.21±0.38a	2.33±0.04a	2.58±0.60	1.95±0.00a
Leucine	7.35±1.91c	5.48±1.55b	5.66±0.58b	4.93±0.08a	5.86±1.48	7.04±0.03c
Phenylalanine	3.62±0.97c	3.36±0.83b	3.19±0.51b	2.16±0.02a	3.08±0.82	3.04±0.02b
Lysine	0.66±0.04b	1.5±0.05d	1.25±0.07c	0.65±0.03b	1.00±0.39	0.21±0.01a

Values followed by different letters in the same row are significantly different (P<0.05).

¹⁾ Values of each genotype (P3, P15 and P22) correspond to the average between both crop years (2012 and 2013) and were compared with the original population (OP) and with white corn (WC) used as control. Parameters expressed and compared on dry basis.

The joint increase of certain essential amino acids in *opaque-2* corn is the product of the combination of biochemical events, such as a higher synthesis and a lower catalysis of these amino acids, an increase in the synthesis and the accumulation of soluble proteins and a decrease in the zein synthesis (Jia *et al.*, 2013). These factors also produce changes in other amino acid content of the kernel. The *opaque-2* genotypes showed higher isoleucine, phenylalanine and threonine average contents with respect to white corn used as control in this study (Table 3). Several research studies have reported higher levels of methionine, cysteine, histidine, isoleucine, leucine, phenylalanine, tyrosine, threonine and valine in whole grains of *o2* and QPM varieties, in relation to normal maize (Ortiz-Martinez *et al.*, 2017).

On the other hand, the contents of arginine, isoleucine and leucine obtained in *o2* progenies (Table 3) were similar to those reported by Rojas-Molina *et al.* (2008) in QPM corn flour. In the same way, the significantly higher levels of aspartic and glutamic acid shown by *opaque-2* genotypes in relation to white corn (Table 3) were coincident with other previous studies (Arruda, Da-Silva and Teixeira, 1978), since these amino acids are structural components of zein. Despite variations in the different amino acid contents of the kernels, the higher protein quality resulting from *o2* mutation in maize is directly associated with an increase in lysine and tryptophan biosynthesis, as well as a change in the endosperm protein composition (Gayral *et al.*, 2016; Ortiz-Martinez *et al.*, 2017).

Lysine content showed a negative correlation with total protein content ($r=-0.54$; $P<0.05$), as reported in other studies (Pereira *et al.*, 2008; Cázares-Sánchez *et al.*, 2015). Likewise, the negative correlation obtained between lysine and zein in this study in both crop years ($r=-0.69$ in 2012 and $r=-0.43$ in 2013; $P<0.05$) was coincident with other reports in which a higher lysine amount in *o2* genotypes led to a reduction in total protein level and therefore in zeins (Landry *et al.*, 2005; Gibbon and Larkins, 2005; Kriz, 2009). Previous research

studies indicated a negative correlation between zein and lysine contents as well as between zein and tryptophan levels, because lysine and tryptophan in maize are directly related (Yau *et al.*, 1999). In addition, the positive correlation obtained between lysine content and albumin fraction ($r=0.81$; $P<0.05$) in the present study would indicate that the higher lysine content of *o2* genotypes was probably due to a higher proportion of mainly albumin levels, in relation to the other non-zein fractions, as reported by Rojas-Molina *et al.* (2008) in nixtamalized QPM corn flours.

Variance components

The genetic variance showed the highest contribution to the total variability obtained in most of the grain characteristics analyzed in this study (Table 4). The lipids in corn kernel are controlled mainly by genetic factors and they are not much affected by the environment and agronomic practices (Arendt and Emanuele, 2013), which could be related to the high genetic variance shown by lipid content (Table 4). However, some studies demonstrated variations in grain lipid content in hybrids cultivated on different plant densities (Scott *et al.*, 2006). A higher contribution of genetic variance was observed in starch content and the effects of crop year and interaction were lower and similar to each other (Table 4). Similar results were reported by Wei, Dai and Liu (2008) in relation to starch and lipid contents.

Similar contributions of the genetic variance and of crop year were obtained in protein content (Table 4). Wei *et al.* (2008) reported similar results in protein content between 10 inbred maize lines. On the other hand, Li *et al.* (2009) obtained low total genetic and additive variances among 14 corn lines indicating a marked environmental influence on this trait. In this study, the contribution of crop year in the protein content was higher than that of genotype x year interaction (Table 4), probably due to the significant high maximum temperatures

Table 4. Relative contribution of genetic (GV), environmental (crop year) (EV) and genotype x year interaction (GxE) variances on the total variability in the studied traits from whole grain flour of *opaque-2* maize genotypes

	Starch	Lipid	Ash	Protein	Protein composition			PD	
					Lysine	Zeins	Non-zeins		
						¹⁾ Z+Z2	AL+GB		G2+G3
GV (%)	67.1	88.9	75.1	51.0	97.3	92.0	64.2	82.7	86.1
EV (%)	15.3	2.7	18.8	45.1	1.7	3.3	32.4	10.6	2.0
GxE(%)	17.6	8.3	6.1	3.8	1.0	4.7	3.4	6.7	11.9

¹⁾ Z+Z2 = zeins; AL+GB = albumins + globulins; G2+G3 = glutelins; PD = in vitro protein digestibility.

recorded during grain filling during the progenies cycle (Table 1).

The genetic variance showed the highest contribution to the total variability in zein content (Table 4), and the same behavior was observed in the non-zein fractions, since the changes in zeins alter the other protein fraction proportions in *o2* mutant genotypes (Landry *et al.*, 2005). The zein content in maize depends on the genotype due to strict control of spatial and temporal expression of the genes that encode them, regardless of culture conditions (Dolfini, Landoni, Tonelli, Bernard and Viotti, 1992; Landry *et al.*, 2005). In the endosperm hardness of QPM germplasm, additive effects are more important than non-additive effects because of the *o2* mutation genetic background (Dolfini *et al.*, 1992), which is closely related to zein content since zeins contribute to endosperm hardness (Dombrink-Kurtzman and Bietz, 1993).

A high contribution of the genetic variance was observed on the *in vitro* protein digestibility. However, the effect of genotype x year interaction was higher than the other protein composition traits (Table 4). Apart from the grain structural characteristics, cooking processes can influence the protein enzymatic digestibility (Duodu *et al.*, 2002).

In the lysine content, the genetic variance contributed to almost all the obtained variability, while effects of crop year and interaction were practically negligible in relation to the genetic effect (Table 4). Other studies have demonstrated similar results (Wegary, Labuschagne and Vivek, 2011). Lysine content of corn kernel is determined mainly by the female parent, indicating an additive effect significantly higher than dominance variance on this trait (Tang *et al.*, 2011).

CONCLUSION

A great variability in physical traits such as grain hardness and the flour chemical composition was found in the genetic materials. The variations of annual averages in centesimal composition show general trends in the proportional changes of the grain starch, protein, lipid and ash contents between generations. The variability in protein composition was related to the differences seen in zein and non-zein protein fractions between *opaque-2* genotypes. Concomitantly, electrophoretic patterns of zein fraction evidenced differences between genotypes; this reflects variability in protein composition and in lysine content and results in a modification in the nutritional quality of grain protein. On the other hand, taking into account that

o2 mutation produces a soft flouy endosperm, which is agronomically unfavorable, in this study some genotypes presented better protein quality and remarkable grain hardness, as was observed in the P13 and P28 progenies.

The higher contribution of genetic variance obtained in zein and lysine contents indicates a marked dependence on genotype, despite the high crop year effect obtained in total protein content. This could indicate a strong additive component in total genetic variance in zein and lysine. However, experiments involving a larger number of environments and sample size are required to obtain a more substantial evidence of the genetic effects on these traits, as the contributions of crop year and genotype x year interaction variances indicate environmental influence.

In vitro protein digestibility of *opaque-2* kernels suggests that their structural characteristics, such as lower zein content, could result in a better protein digestibility, which would be relevant from a nutritional point of view given its high protein quality. However, studies based on protein bioavailability are required to obtain a more accurate nutritional evidence.

The differences found in the protein composition of *opaque-2* genotypes would facilitate the identification of individual families with lower zein proportion and higher lysine content for their use in subsequent selection cycles. It would also enable researchers to continue improving and developing varieties with specific traits for production of processed food with higher nutritional quality.

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