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Original Paper

Assessment of Protein Quantity and Quality of Turkish Maize Landraces with Different Opacity Levels

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Protein ratio and amino acid composition vary considerably in maize according to the level of opacity. In this study, the total protein content, amounts of some essential amino acids (lysine and tryptophan) and quantitative and qualitative variation of protein fractions in maize seed samples separated according to different opacity levels were investigated. In the study, 6 local maize landraces with opaque kernel structure and 3 standards were used. The field trial was carried out in 2021 according to the complete blocks trial design with 3 replications. Samples of local maize landraces from the field experiment were separated on a light table according to 5 different opacity levels (0%, 25%, 50%, 75% and 100%). Protein content, lysine and tryptophan contents, albumin + globulin, glutelin and zein contents were analyzed in the samples separated by their opacity. In addition, protein fractions were subjected to SDS-PAGE analysis and the changes in protein bands according to opacity level were examined. Differences between genotypes, opacity levels and their interaction were illustrated with box plot and PCA-Biplots. Two-way dendrograms were created for qualitative discrimination. The results of the study showed that there were significant changes in protein amount and quality according to the opacity levels of the genotypes. The protein contents of the genotypes were 5.95–10.22%, lysine contents 0.33–0.46%, tryptophan contents 0.032–0.083%, albumin + glubulin contents 1.92–2.72%, glutelin contents 0.64–1.08%, and zein contents 1.59–1.99%. There was a significant difference in presence or absence according to the opacity levels in the protein fractions.

Keywords: essential amino acid, *Zea mays*, genetic resources, food

1 Introduction

Maize is one of the staple grains, each part of the plant has economic value and it is included in the industry as human food or animal feed. While the world production of maize was 792 million 732 thousand tons in 2007, it increased to 1,149 billion tons in 2019 (Özcan, 2009; Anonymous, 2020). In Turkey, the cultivation area of maize was 692 thousand hectares and the production amount was 6.5 million tons in 2020 (Anonymous, 2021). Maize contains 8–10% protein and 75% of proteins are found in the endosperm, 22% in the embryo, 2% in the husk and 1% in the pedicel (Yıldırım et al., 2020). Grain quality is as important as protein content and protein fractions have an important effect on the variations in protein quality. There are four protein fractions in maize based on solubility, namely albumin, globulin, glutelin and zein. The zein fractions that predominate in maize are generally poor in essential amino acids such as lysine and tryptophan (Wu et al., 2010). Most of the storage proteins in maize grain are composed of zein fractions, which reduces the protein quality of maize and limits its use for human and animal nutrition (Duvjnak et al., 2021).

Improving quality of maize is one of the goals in many breeding programs (Egesel and Kahrıman, 2012). One of the most common goals of breeding for grain quality is to improve protein quality. Protein quality in maize is closely related to the protein fractions found in the endosperm. The protein quality of maize was increased by the recessive opaque2 (o2) mutant, which led to the improvement of protein quality in maize, and by endosperm modifications, as well as the development of Quality Protein Maize (QPM) (Egesel and Kahrıman, 2012). Scientific studies show that the opaque2 gene has an important effect in improving protein quality.

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The homozygous o2 mutant reduces the amount of zein in the endosperm and increases the lysine and tryptophan content to higher levels and this mutation causes an increase in protein fractions, apart from zein (Gibbon and Larkins, 2005).

Improvement of protein quality in maize is possible with a breeding strategy to increase essential amino acids (Prasanna et al., 2001). For this reason, it has become necessary to search for genetic resources that can enable the development of high protein quality maize and to identify promising varieties (Pfunde et al., 2015). Various research results demonstrated that high protein quality materials can be developed depending on the opacity level of maize germplasm. Some studies were conducted on the protein fractions of local maize populations in Turkey (Akbulut et al., 2021.). However, when compared with studies on local maize landraces of other countries, available studies are limited in scope. This study was carried out to investigate the differences in protein ratio, lysine and tryptophan contents and protein fractions in seed samples with different levels of opacity from Turkish local maize populations, which were previously selected for their opacity characteristics, and to determine the genotypes that can be used as source material in breeding studies.

2 Material and methods

2.1 Field experiment

The field experiment was conducted in Sarıcaeli Village in Çanakkale province in the summer growing season of 2021. Materials for the research study comprised 6 opaque local maize populations (POP1, POP2, POP3, POP4, POP5, POP6) and 3 standard genotypes (2 normal,1 opaque).

The field experiment was established according to randomized complete block design. Each genotype was sown at a spacing of 70 × 20 cm in two-row plots with a row length of four meters. Sowing was done manually on May 18, 2021. Weed control was practiced manually in the experimental area. Irrigation was performed weekly using the drip irrigation system installed in the experiment area. Fertilization was administered through the drip irrigation system and was applied based on the results of soil analysis.

2.2 Soil and climatologic fFeatures of experiment area

The results of soil analysis from the field before the experiment are shown in Table 1. In terms of soil properties, the experimental area was poor in terms of organic matter and high in clay content (Table 1). Çanakkale province is located in the northwest of Turkey. Since the area where the experiment was conducted

Table 1 Materials used in the experiment

Soil Analysis	Results	Classes
pH	7.39	neutral
Soil properties (%)	67.7	clay-loam
E.C. (mS.cm^{-1})	89.3	low
Organic matter (%)	1.87	low
Lime (%)	8.50	medium chalky
P (kg.da^{-1})	2.83	low
K (kg.da^{-1})	80.80	low

Table 2. Climatological conditions in the experiment area.

	May	June	July	Aug	Sep	Oct
Average Temp.	19.90	24.10	28.20	28.30	23.10	18.10
Total Rainfall	57.3	57.1	2.0	0.0	8.9	75.9
Max Temp	36.6	38.5	39.1	39.7	32.8	24.6
Min Temp	11.2	13.8	19.8	21.5	12	11
L.Y. Temp.	17.51	22.21	25.02	24.98	21.05	16.18
L.Y. Rainfall	30.1	24.6	11.7	6.6	22.8	54.1

is close to the center of the province, it has similar climatic characteristics. The average temperature during the months of the experiment was similar to the average temperature over many years (Table 2). However, the temperature was higher than the long term values. Summer is generally dry and there is no precipitation in August. Fertilizer was administered on June 23, 2021, and the main fertilizer (urea) was applied to the rows with 10 kg of nitrogen/da and mixed with soil.

2.3 Laboratory Analyses

2.3.1 Preparation of samples

The seed samples were obtained from the experimental field and then separated according to five levels of opacity (0%, 25%, 50%, 75% and 100%) for each population using a light table. This separation was performed and then the separated seeds were ground in a laboratory mill using a sieve with diameter 0.5 mm. The ground samples were stored at a temperature lower than +4 °C for further laboratory analysis.

2.3.2 Total protein content

The protein content of the samples was determined using a benchtop NIR instrument (SpectraStar 2400 D, Unity Scientific, USA). For this purpose, the soil samples were scanned in the fixed cup mode of the instrument and the spectral data were used in a local calibration model (Egesel and Kahrıman, 2012) to determine protein values in dry matter.

2.3.3 Lysine and tryptophan analyses

For lysine and tryptophan analyses, the samples were subjected to extraction in a Soxhlet device for 6 hours to remove crude oil from 10 g of samples separated according to the opacity level of each genotype. Subsequently, lysine and tryptophan analyses were performed according to the method proposed by Galicia et al. (2009). Both amino acids were determined colorimetrically using a microplate reader (Biotek Epoch, Agilent Technologies, USA).

2.3.4 Determination of protein fractions

The protein fractions in maize were obtained as follows: albumin-globulin used 0.5 N NaCl solution, 70% ethanol and 2% β-mercaptoethanol was used for glutelin, and 1% SDS and 2% β-mercaptoethanol solutions were prepared for zein. Endosperm meal was weighed to 100 mg on a precision balance. The extraction solutions were added to the samples in the tubes, shaken on an orbital shaker for 1 hour and extracted twice. Albumin and globulins were extracted at 4 °C, zein and glutelin at 22 °C. Mixtures of sample and extraction buffer were centrifuged at 10,000 g for 10 minutes, and the supernatants were removed and transferred to tubes. In this way, the relevant protein fractions of maize endosperm were isolated from the homogenate. These extracts were stored at +4 °C for use in SDS-PAGE gel analysis and quantitative quantification (Yau et al., 1999).

For quantitative determination of protein fractions, albumin + globulin and glutelin were determined colorimetrically according to the Bradford method (Bradford, 1971). The prediction equation (R^2 = 0.99) was used with the standard curve prepared for BSA. For zein differentiation, the curve (R^2 = 0.99) prepared with a standard substance (Acros Organics, Zein purified) was used.

2.3.5 SDS-PAGE Analyses

SDS-PAGE was used for qualitative separation of protein fractions (Yau et al.,1999). In these analyses, 15% solvent gel and 4% separation gel were prepared for albumin-globulin or glutelin. For zeins, 12% solvent gel and 4% separation gel were prepared. Prior to loading the gel, 150 microliters of 4X loading buffer was added to 50 microliters of sample. After vortexing, the samples were placed in a water bath at 95 °C for 5 minutes. At room temperature, 7 microliters of sample and molecular weight standard (Thermoscientific, PageRuler Plus Prestained Protein Ladder No:00745695.) were loaded into the gel wells using a Hamilton syringe. The gels were run at 120 V until complete separation of the samples (approximately 6 hours). The gels were then removed from the gel tank and placed in a mixed solution of 60 g TCA, 1 g Brilliant Blue and 25 mL ethanol with distilled water to 500 mL and left on the shaker for approximately 6 hours and stained. The images of the gels were then captured and transferred to the Gel Analyzer program (www.gelanalyzer.com) for examination. The 9 bands formed by the standards loaded with the samples were identified and their molecular weights were defined as 10, 15, 25, 35, 55, 70, 70, 100, 130, and 180 kDa. The molecular weights of the bands formed by the other samples, corresponding to the standards whose molecular weights were defined, were calculated by the program and their presence in the bands was coded in 1 and 0 format and transferred to an Excel file.

2.4 Statistical analysis

The data obtained from the study were analyzed in R software (R Core Team, 2019). Analysis of variance was used to examine the changes for genotypes, opacity and their interactions. Boxplots were created for each factor to compare differences among factor levels. PCA-Biplot was created to show interaction effects of factors on investigated traits. Qualitative data for protein fractions

were subjected to heatmap dendrogram using the heatmap package.

3 Results and discussion

Changes in protein, lysine, tryptophan ratios and protein fractions according to level of opacity are shown in Figure 1. Although there were significant differences in protein content according to the levels of opacity, where the highest values were obtained at 25% and 50% opacity and the lowest protein content was observed at 100% opacity. Sevenayak and Gupta (2017) conducted an experiment on three different genotypes (regular, opaque and QPM). According to the results of the study, the highest protein content was found in QPM maize while the lowest protein value was found in opaque maize. The normal maize genotypes were between these two groups. In our study, the decrease in protein content with increasing opacity level supports these results. When the relationship between lysine content and opacity is examined, the highest value was calculated at 75% opacity and the lowest value was calculated at 0% opacity. It is well known that the nutritional quality of corn varies depending on lysine and tryptophan contents. Sevenayak and Gupta (2017) selected three different genotypes (regular, opaque, and QPM) to observe the lysine and tryptophan contents. According to their observations, lysine and tryptophan contents were highest in opaque maize and lowest in regular maize. Tryptophan content was highest at 100% opacity and lowest at 0% opacity. Pukalenthy et al. (2020) used a marker-assisted backcrossing (MABC) strategy to increase lysine and tryptophan content. They worked with the SSR marker umc1066 during gene transfer. The

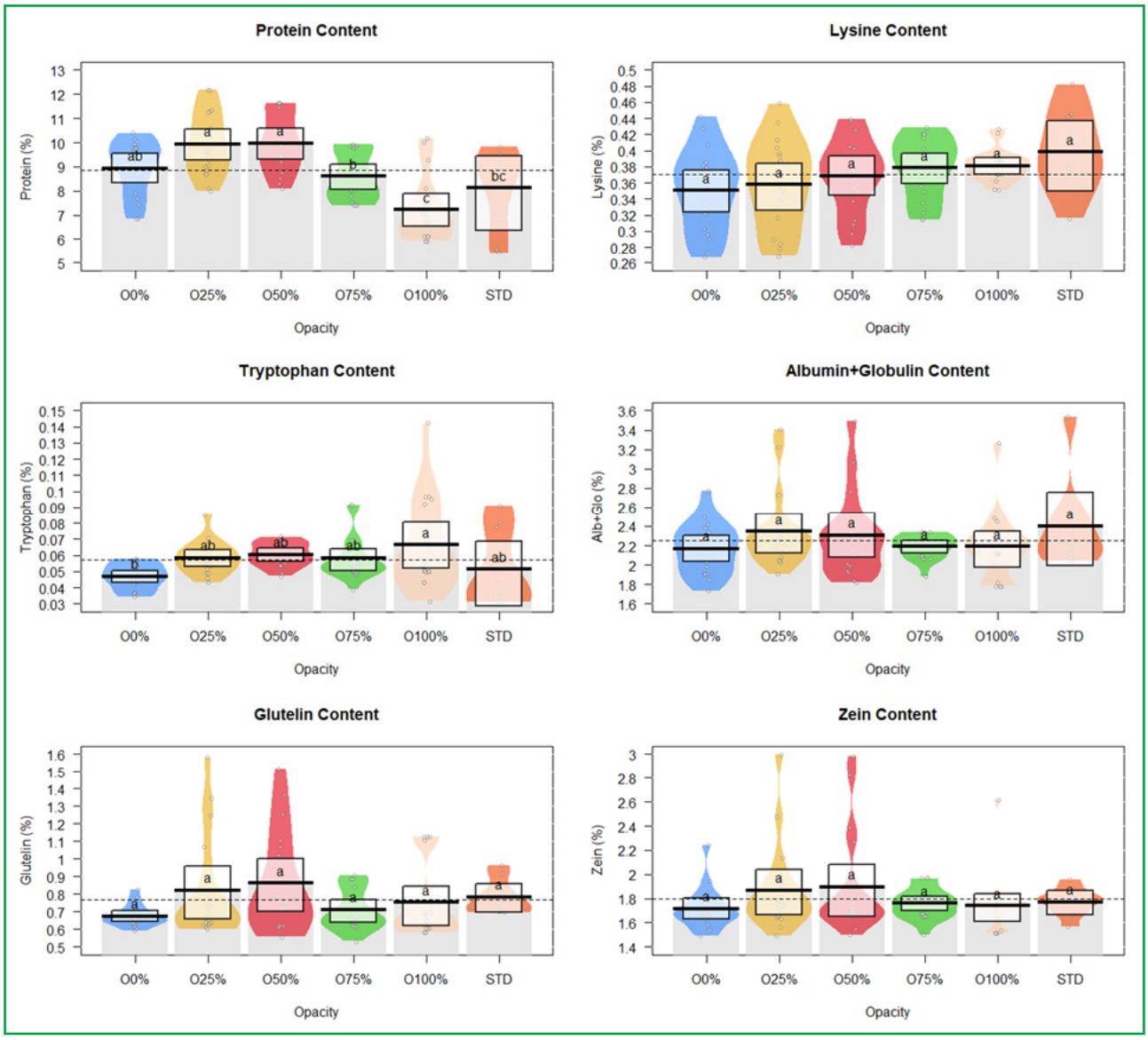

Figure 1 Variation in the properties examined based on levels of opacity

lines improved by gene transfer had better agronomic performance with increased lysine (0.311–0.331%) and tryptophan (0.040–0.048%) contents. The marker-assisted backcrossing breeding strategy successfully improved lysine and tryptophan levels without affecting agronomic performance. Albumin globulin content was highest at 25% and 50% opacity and lowest at 75% opacity. Sethi et al. (2021) conducted a study to determine whether grain maturity would increase albumin globulin content. The results of study showed that the maturity level of grain varies with the amount of prolamin and glutelin. Accordingly, as the amount of prolamin and glutelin decreases, the amount of albumin-globulin increases in the same way. If the changes in glutelin content are examined, the highest values were found at 50% opacity and the lowest values were found at 0% opacity. Sethi and Chaudhary (2019) used two main lines, normal and QPM (opaque mutant), in a study about prolamin and zeins. According to the results of the study, the amount of prolamin was highest in the normal line (44.9%) and lowest in QPM (8.94%), while the amount of glutelin was higher in QPM (32.9%) than in normal lines (17.6%). Different levels of opacity also caused significant changes in zein content. The highest values were observed at 50% opacity. The lowest zein content was obtained at 0% opacity.

Wall and Bietz (1987) described the differences in endosperm proteins between normal and opaque-2 maize. In this study, the effects of opaque-2 mutation on the composition of maize endosperm proteins and their effects on relative synthesis rates during seed development were determined by SDS-PAGE and two-dimensional electrophoresis. Protein-free nitrogen,

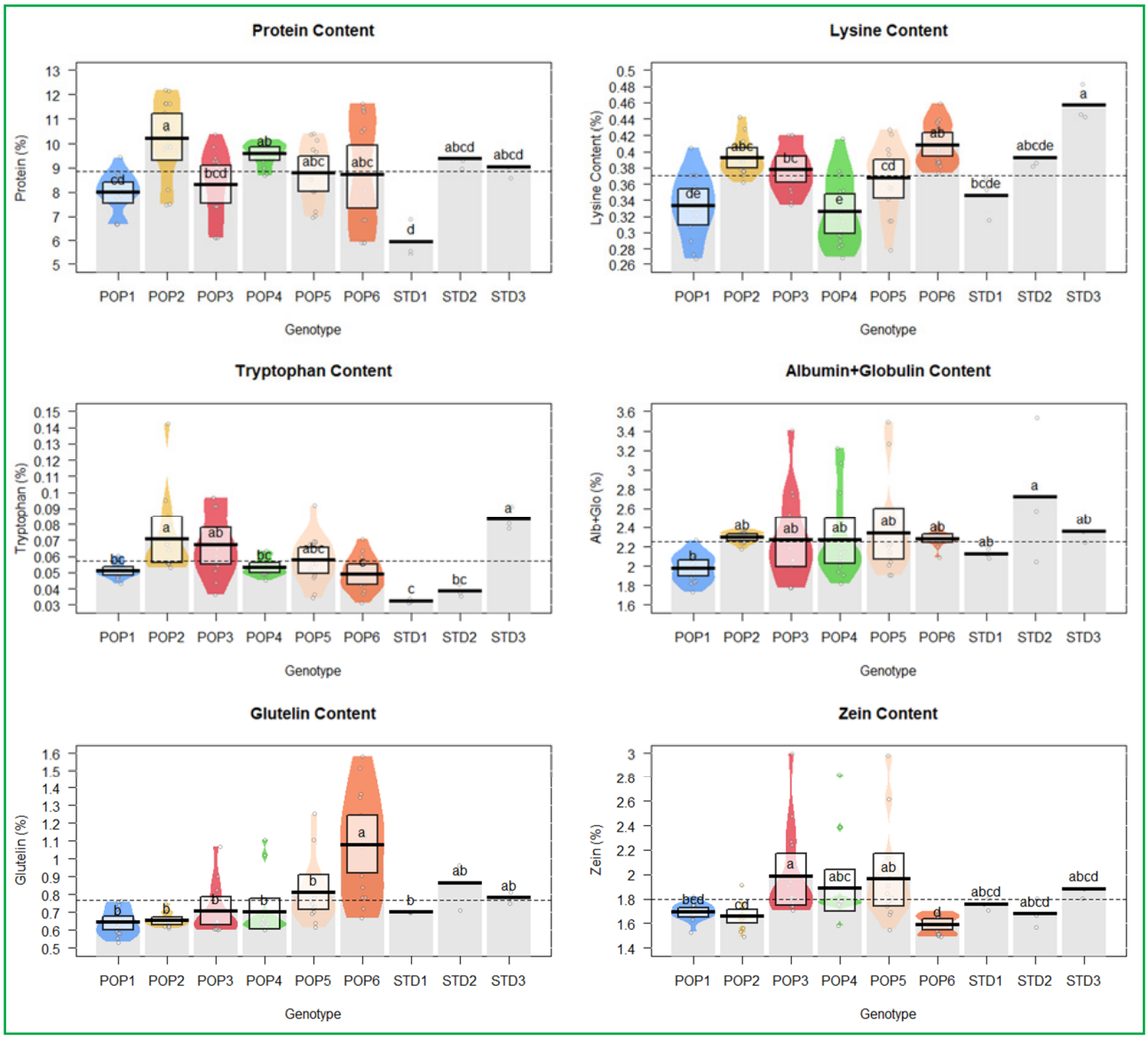

Figure 2 Variations in traits studied according to genotypes

albumins and globulins, zeins, alcohol-soluble reduced glutelins (ASG), and alcohol-insoluble reduced glutelins (AIG) were sequentially extracted from ground endosperm obtained by crushing maize kernels harvested 18, 22, 30, and 48 days after pollination. In the early stages of the normal genotype, high levels of protein-free nitrogen, albumins, and globulins were observed. In the opaque-2 grain, the zein level was not as high and the final AIG level was higher than that of normal endosperm. The SDS-PAGE method showed that albumins and the globulins were reduced during maturation of both normal and opaque-2 kernels.

Figure 2 shows the protein, lysine, tryptophan, albumin-globulin, glutelin and zein contents according to the populations. The protein content was highest in the POP2 genotype and significantly lowest in the STD1 genotype. The highest value for lysine content was calculated in STD3 genotype and the lowest value was calculated in POP4 genotype. STD 3 genotype is the standard with 100% opacity. If the tryptophan content is examined, POP2 and STD3 genotypes had the highest values. STD1 and STD2 genotypes with 0% opacity had the lowest tryptophan content. STD1 and STD2 genotypes have 0% opacity. Albumin-globulin levels were also quite different. The highest albumin-globulin level was observed in STD2 genotype with 0% opacity. The lowest albumin-globulin level was recorded in POP1 genotype. For glutelin content, POP6 genotype had the highest value with a high difference. The lowest value of glutelin was observed in POP1 and STD1 genotypes. For another trait, zein, the highest value was noted in POP3 genotype while the lowest value was in POP6 genotype.

The PCA-Biplot plot showing the opacity *x* genotype interaction for the six traits is presented in Figure 3. In the PCA-Biplot plot, 54.2% of the total variation in the traits was explained. Considering the directions of the vectors of the traits, zein and alb + glob content and other lysine, tryptophan and glutelin contents were negatively correlated traits. Among the samples, samples with 50% and 75% opacity from the P6 coded population and STD3 (opaque standard) genotypes were found to have higher tryptophan, glutelin and lysine contents than the other samples. The protein content of these samples was also higher than the other samples. In terms of albumin+glubulin content and zein content, high values were determined in samples with 50% opacity from P3 genotype and 75% opacity from P4 genotype. Considering the distribution of the symbols for the samples with different opacity levels on the graph, the samples with 75% opacity and 100% opacity were clustered in certain regions and the shapes of the ellipsoids around these samples confirm the increase in protein quality with the increase in opacity level (Figure 3).

3.1 SDS-PAGE analysis of Protein fractions

The dendrograms for the protein fractions are shown in Figure 4, Figure 5 and Figure 6. In the dendrogram for the albumin+glubulin fractions, the protein bands were grouped into two clusters. The first cluster contained 4

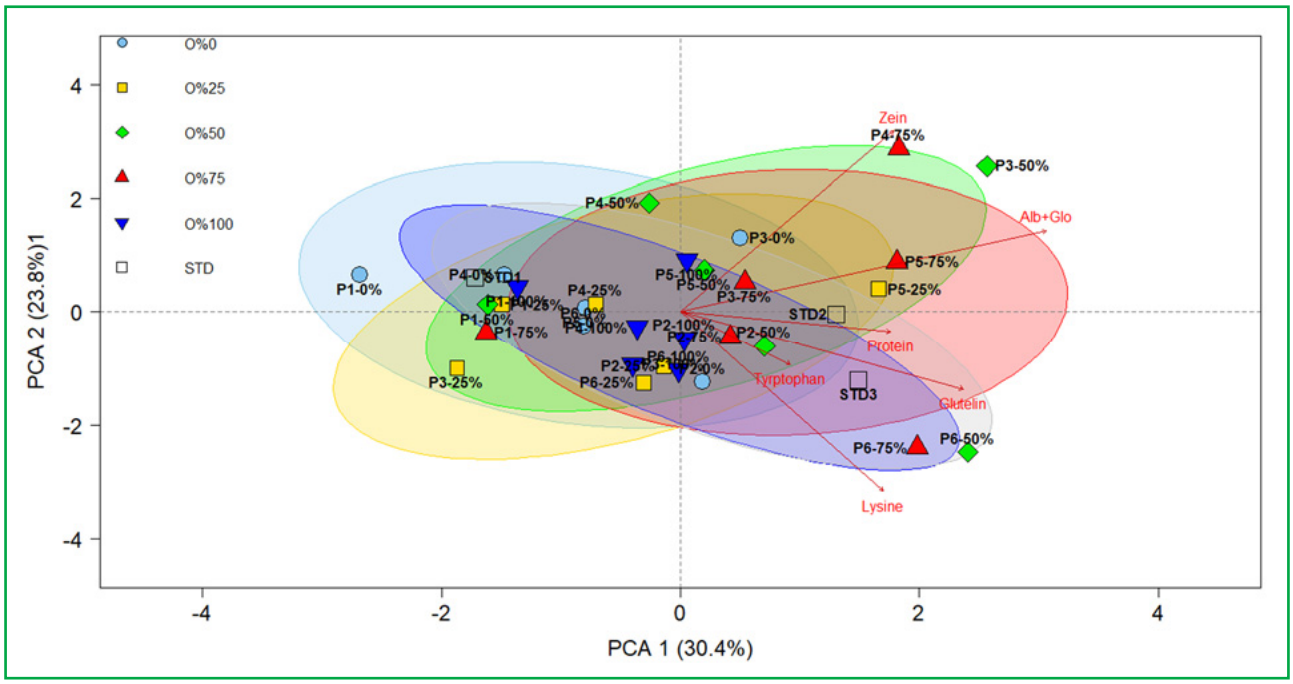

Figure 3 PCA-Biplot graphic for investigated traits

bands (MW12, MW20, MW15 and MW21) and 6 bands (MW52, MW35, MW25, MW10 and MW40). In this group, high molecular weight bands were generally observed. Looking at the horizontal clustering of the genotypes, there were basically 2 sub-clusters. While 11 genotype samples were grouped in the first cluster, 22 samples were grouped in the second cluster. It is noteworthy that the bands separating these groups were the MW25, MW10 and MW40 bands, which were present in the first cluster (yellow areas) while they were generally absent in the second (black areas). Although no clear distinction was observed in terms of grouping by opacity level, it was noteworthy that some protein bands were present in all samples with high opacity levels. For example, the MW21 band was present in all samples a 100% opacity level (Figure 4). In their study, Sethi et al. (2019) found that prolamins and glutelins could be used as markers to distinguish normal lines from QPM lines in dendrogram analysis based on the group mean of experimental genotypes. The dendrogram analysis also showed that genetic background plays an important role in modifying the nutritional quality of maize.

In the dendrogram for glutelin fractions, the protein bands were divided into 2 groups. The first group contained 20 samples with different levels, while the second group contained 13 samples with different levels of opacity. The first group in the rows generally contained genotypes with 50% opacity, while the second group contained genotypes with 100% opacity. Looking at the columns, 2 groups were observed. Group 1 in this

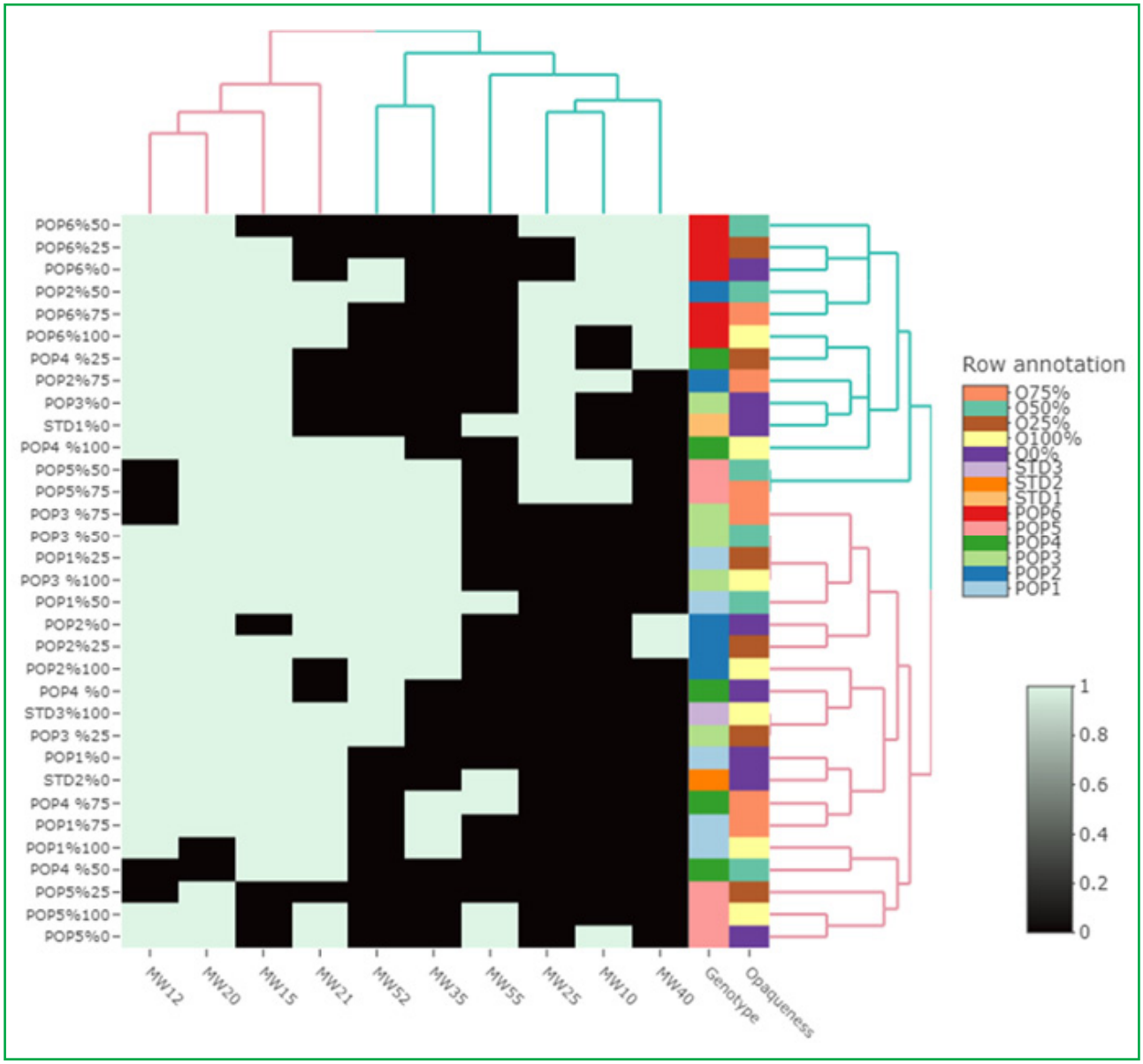

Figure 4 Heatmaps for albumin + globulin fractions

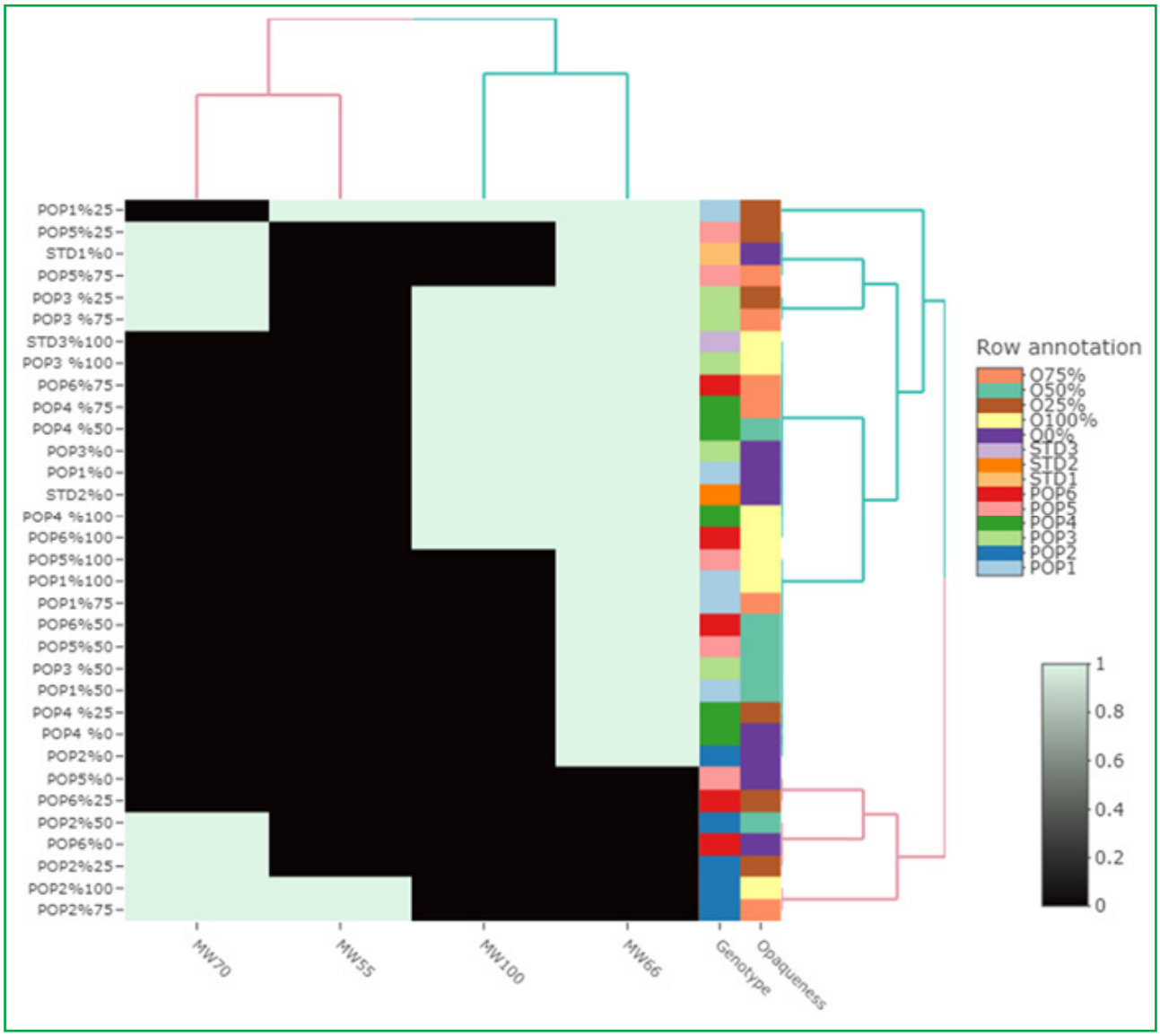

Figure 5 Heatmaps for glutelin fractions

column included MW70 and MW55 bands, while group 2 included MW100 and MW66 bands (Figure 5).

In central Mexico, Aguirre-Mancilla et al. (2019) conducted a study on the protein quality and content in the seeds of some maize varieties. They concluded that the molecular weight of the missing prominent band in the middle of gel (45 kDa) of accession '12013' varied. This band could help to distinguish the differences between the accessions. In addition, in the electrophoretic output of the prolamin fraction, while the normal maize accessions coded 'FVR2015', 'ERH2015', 'HRH2015' and 'MRH2015' has similar profiles to each other, the QPM maize accession coded 'INIFAP-QPM' had a band at 33.1 kDa, which was not found in other accessions.

The protein bands were divided into two groups on the dendrogram for zein fractions (Figure 6). In terms of rows, again 2 groups were observed. Group 1 included all genotypes with diftferent opacity levels except 1 genotype. Group 2 included only the POP2 genotype at 25% opacity. The columns show 2 clusters. In the first cluster, MW35, MW26, and MW25 bands (yellow areas) were observed to contain the POP2 genotype at 100% opacity. In second cluster, MW24 and MW23 bands were observed.

Sarika et al. (2018) studied an endosperm-modified opaque maize population. In the study, they performed quantitative analysis using the SDS-PAGE method to compare the prolamin fraction. With this analysis, they confirmed that the o16o16 (QPM) genotypes had similar profiles to the normal line and similar grain hardness to the wild normal maize line CML543. However, the modified o2o2 genotype showed a twofold increase in γ-zein expression at 16–27 and 50 kDa, and they

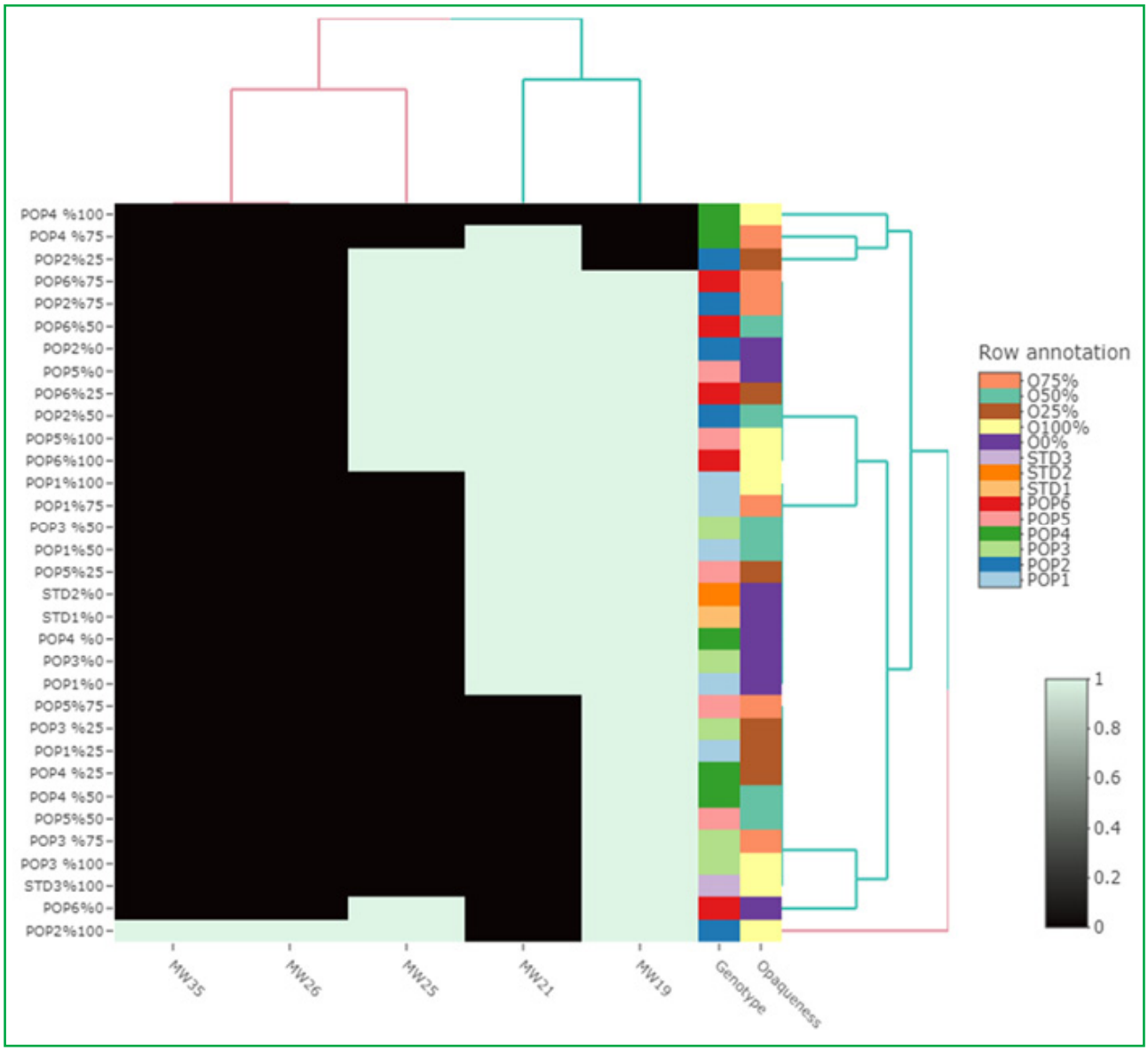

Figure 6 Heatmaps for zein fractions

identified this twofold increase as the main factor in endosperm modification.

4 Conclusions

The results of this study showed that the increase in opacity caused a decrease in protein ratio in opaque maize. It was observed that the content of lysine and tryptophan, essential amino acids, increased proportionally to the increase in the opacity level of the samples. Among the genotypes, POP2 coded genotypes differed from other genotypes in terms of both protein content and amino acid content. However, genotype x opacity interaction was found to be significant for most of the examined traits. The results of the SDS-PAGE analysis showed that some of the protein band fractions can help discrimination according to the opacity level of the genotypes, but a clear distinction cannot be achieved for all fractions. In electrophoresis analysis, most of the samples, especially with 100% opacity, were clearly separated compared to other opacity levels. In future studies, the use of techniques that can provide more precise separation of opacity level and performing laboratory tests with more sensitive techniques may provide further details and more precise information.

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