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### **KERNEL MODIFICATIONS AND TRYPTOPHAN CONTENT IN QPM SEGREGATING GENERATIONS**

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Maize has poor nutritional value due to deficiency of two essential  
amino acids – tryptophan and lysine. Although recessive *opaque2* (*o2*)  
mutation significantly increases their content in the endosperm,  
incorporation of *opaque2* into high yielding cultivars was not commercially  
successful, because of its numerous agronomic and processing problems  
due to soft endosperm. Quality protein maize - QPM has lately been  
introduced as *opaque2* maize with improved endosperm hardness and  
improved agronomic traits, but mostly within tropical and subtropical  
germplasm. The ongoing breeding project at MRI includes improvement of  
MRI *opaque2* lines and conversion of standard lines to QPM germplasm.

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The main selection steps in QPM breeding involve assessing kernel modifications and tryptophan level in each generation. Herein, we present the results of the analysis for these traits on F3 and BC1F1 generations of QPM x *opaque2*, *opaque2* x QPM and standard lines x QPM crosses. The results showed that the majority of the genotypes had kernel types 2 and 3 (good modifications). The whole grain tryptophan content in F3 and BC1F1 genotypes of crosses between QPM and *opaque2* germplasm was at the quality protein level, with a few exceptions. All BC1F1 genotypes of standard lines x QPM had tryptophan content in the range of normal maize, while majority of F3 genotypes had tryptophan content at level of QPM. The progeny (with increased tryptophan levels) of QPM and *opaque2* crosses had significantly higher tryptophan content compared to the progeny of crosses between standard and QPM lines – 0.098 to 0.114 and 0.080, respectively. All genotypes that had poorly modified kernels and/or low tryptophan content will be discarded from further breeding.

*Key words:* kernel modifications, maize, *opaque2*, QPM, tryptophan content

## INTRODUCTION

Maize is one of the most important crops in human and animal nutrition. However, like most other cereals, its nutritional value is poor due to deficiency of two essential amino acids – tryptophan and lysine. It was shown that *opaque2* (*o2*) mutation can nearly double the lysine content of the endosperm compared with the wild type (MERTZ *et al.*, 1964; EGGUM *et al.*, 1983; EGGUM *et al.*, 1985). Maize homozygous for the *o2* (recessive) mutation has substantially higher lysine and tryptophan content than maize that is either homozygous dominant (*O2O2*) or heterozygous (*O2o2*) for the *opaque2* locus (CROW and KERMICLE, 2002). BRESSANI (1992) showed that increased concentrations of these two amino acids in the grain endosperm can double the biological value of maize protein. The biological value of QPM protein is about 80% that of milk is about 90% and that of normal maize 45% (FAO 1992).

Incorporation of *opaque2* into high yielding cultivars was not commercially successful, because of its numerous agronomic and processing problems – reduced grain yield, soft endosperm, chalky and dull kernel appearance and susceptibility to ear rots and stored grain pests. These drawbacks have been corrected in genetically improved, hard endosperm quality protein maize – QPM (VIVEK *et al.*, 2008). QPM is a genotype in which *opaque2* has been incorporated along with associated modifiers - the endosperm hardness modifier genes that convert the soft/opaque mutant endosperm to a hard/vitreous endosperm and modifier/enhancers that confer higher tryptophan and lysine content. Thus, breeding of QPM varieties requires manipulation of three different genetic systems (KRIVANEK *et al.*, 2007).

Potential contribution of QPM to human nutrition, especially in poor countries where maize is staple food, has been well documented (BRESSANI, 1991; AKUAMOA-BOATENG, 2002; MORALES-GUERRA, 2002). The significance of QPM is

obvious from the fact that in 1977 only four countries grew QPM, but in 2003 more than 23 countries have realised QPM varieties for large scale cultivation on area over 3.5 million hectares (SOFI *et al.*, 2009). Although QPM development was primarily targeted at developing and under-developed countries, its production and consumption in developed countries could also be advantageous. Application of QPM as animal feed can be very beneficial (increased weight gain) especially for monogastric animals such as pigs and poultry, which require a more complete protein than cereals alone can provide. In Brazil and El-Salvador use of QPM in animal feed reduced use of soybean meal by about 50% besides reducing use of synthetic lysine supplements (LOPEZ-PEREIRA, 1992).

QPM germplasm is of tropical and sub-tropical origin and its adaptation to temperate climatic region is a challenging task. Maize Research Institute (MRI) maintains a collection of *opaque2* inbred lines developed in the seventies (DENIĆ *et al.*, 1979; DENIĆ *et al.*, 1984; DRINIĆ *et al.*, 2007; KONSTANTINOV and MLADENOVIC DRINIC, 2007). Recently, these lines have been analyzed for protein and tryptophan content and are used for breeding QPM (IGNJATOVIĆ-MICIĆ *et al.*, 2008; IGNJATOVIĆ-MICIĆ *et al.*, 2009). Besides good agronomic performances, there are two unique and essential steps in the development of QPM germplasm (VIVEK *et al.*, 2008). The first is to simultaneously identify segregants in a family or population having the *o2* allele in the homozygous recessive (*o2o2*) condition with a hard endosperm, through kernel modification score. The second step is to identify and confirm the presence of the essential amino acids, i.e. percentage of tryptophan in a sample. The breeding project at MRI involves QPM x *opaque2*, *opaque2* x QPM and standard lines x QPM crosses, with the aim to improve MRI *opaque2* or convert standard lines to QPM germplasm. Following the selection steps, choice of the material for further selection was based on kernel modification score and tryptophan level in each generation. Herein, we present the results of the analysis of F3 and BC1F1 generations for kernel modification and tryptophan content for all three types of crosses.

## MATERIALS AND METHODS

The material analyzed for kernel modifications and tryptophan content consisted of F3 and BC1F1 progeny of three different types of crosses - QPM x *opaque2*, *opaque2* x QPM and standard lines x QPM. Four QPM lines (CML144, CML159, CML161 and CML165 from CYMMIT, Mexico), two QPM varieties (SussF and Mat-pro from IIAM, Mozambique) and 15 *opaque2* lines (from MRI, Serbia) were used for producing QPM x *opaque2* genotypes (28 F3 and 19 BC1F1) and *opaque2* x QPM genotypes (20 F3 and 15 BC1F1). F3 (9 genotypes) and BC1F1 (19 genotypes) generations of standard lines x QPM were obtained by crossing four standard inbred lines (ZPL175, B73, 255/75-3 and 255/75-5) with CML144, CML159, CML161, CML165, SussF and Mat-pro.

Kernel modification was visually assessed using light table, according to the scoring scale from 1 (completely translucent, with no opaqueness) to 5 (completely opaque). Modification score 2 is given to the kernel which is 25%

opaque, while scores 3 and 4 are given to 50% and 75% opaque kernels, respectively.

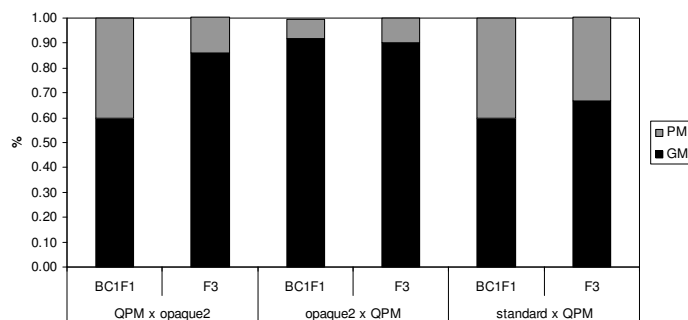
Tryptophan content was determined according to NURIT *et al.* (2009). The protocol is based on the Hopkins-Cole reaction, in which one molecule glyoxylic acid and two molecules tryptophan form a colored compound with a maximum absorption at 560nm. Briefly, random samples of 30 seeds were grounded to a fine powder, defatted and digested with papain. Colorimetric reaction was developed using  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  dissolved in glacial acetic acid and 30N  $\text{H}_2\text{SO}_4$ . Absorption was read at 560nm in a spectrophotometer. Tryptophan % was calculated using a standard (calibration) curve, developed with known amounts of tryptophan, ranging from 0 to 30 $\mu\text{g/ml}$ .

## RESULTS AND DISCUSSION

### Kernel modifications

Kernel modification scores for F3 and BC1F1 generations of all three cross types are presented on Graphic 1. Both type 2 and type 3 kernels (good modifications) can be used for further selection, while type 4 and type 5 (poor modifications) are discarded. Genotypes were scored according to the majority of the kernel modification – if most of the seeds were scored 3, and a small fraction was scored 4 or 2, than the whole genotype was scored 3.

The best modifications were recorded among *opaque2* x QPM BC1F1 and F3 genotypes, i.e. 8% and 10% genotypes had poor modifications (mostly type 4 kernels). The highest number of genotypes with poorly modified kernels was found in BC1F1 of QPM x *opaque2* crosses, i.e. 40%, while in the F3 genotypes of the same type of crosses 85% of the genotypes had good and only 14% poor modification scores. In F3 genotypes of standard line x QPM crosses 67% of genotypes had type 3 and type 2 kernels and in BC1F1 60% of genotypes had kernels with good endosperm modifications.

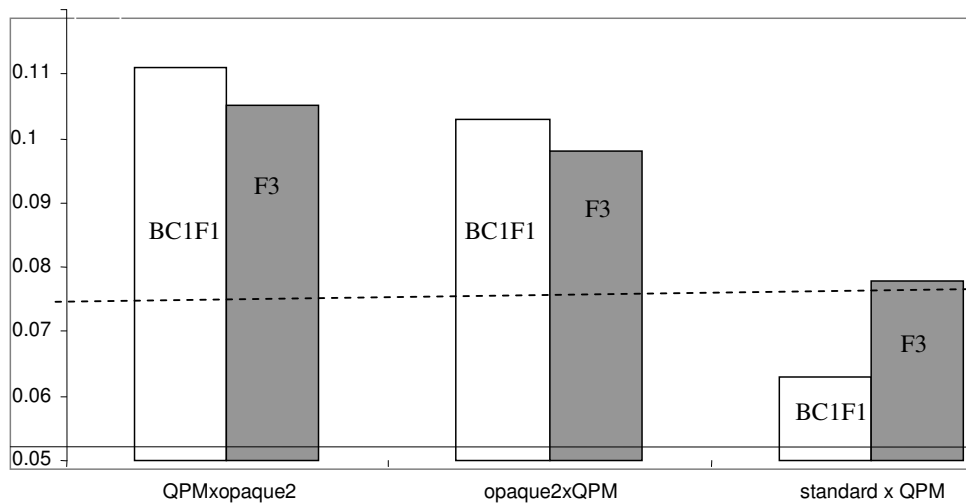


Graphic 1. Kernel modifications in different types of crosses and generations

Legend: PM – poor modifications (kernel types 4 and 5) and GM – good modifications (kernel types 2 and 3)

**Tryptophan content***Crosses between QPM and opaque2 germplasm*

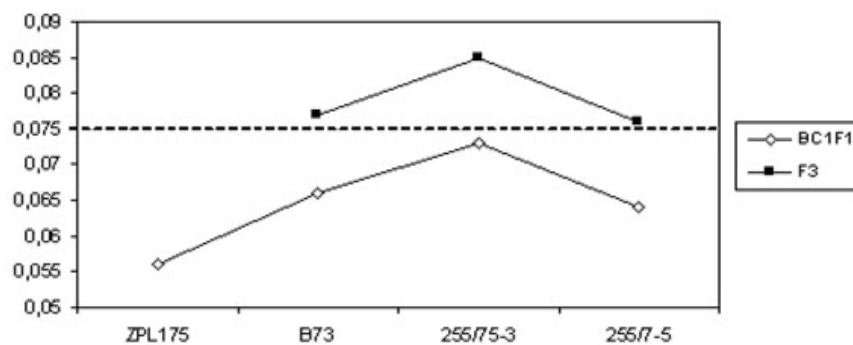
The average tryptophan content (%) in whole grain (ATC) of different types of crosses and generations are presented on Graphic 2. In both QPM x *opaque2* and *opaque2* x QPM germplasm ATC was significantly higher than the limit of 0.075 set for QPM germplasm. There were no significant differences between BC1F1 and F3 generations – values were 0.111 and 0.105 for QPM x *opaque2* and 0.103 and 0.098 for *opaque2* x QPM, respectively. Overall, ATC was slightly higher in QPM x *opaque2* compared to *opaque2* x QPM – 0.111 vs. 0.103 in BC1F1 and 0.105 vs. 0.098 in F3, respectively. Also, in both types of crosses ATC was slightly higher in BC1F1 vs. F3. All these differences can be considered negligible as they are in the range from 0.005 (between BC1F1 and F3 generations of QPM x *opaque2*) to 0.008 (between BC1F1 generations). Among all analyzed genotypes, only CML144 x P802 in F3 had tryptophan content below the 0.75 limit, i.e. 0.064. Within both cross types and generations in all the other genotypes tryptophan content ranged from 0.078 to 0.146.



Graphic 2. Whole grain average tryptophan contents (%) in different types of crosses and generations (dashed line – tryptophan threshold level for QPM)

*Crosses between standard and QPM lines*

Tryptophan content in F3 and BC1F1 generations of standard lines x QPM crosses showed significant differences. ATC for all crosses in BC1F1 genotypes was below the QPM limit - 0.064 and in F3 genotypes over the QPM limit – 0.078. The lowest ATC was determined in BC1F1 progeny of ZPL175 and the highest in F3 progeny of 255/75-3 (Graphic 3).



Graphic 3. Whole grain average tryptophan content (%) in BC1F1 and F3 progeny of different crosses between standard and QPM lines (dashed line – tryptophan threshold level for QPM)

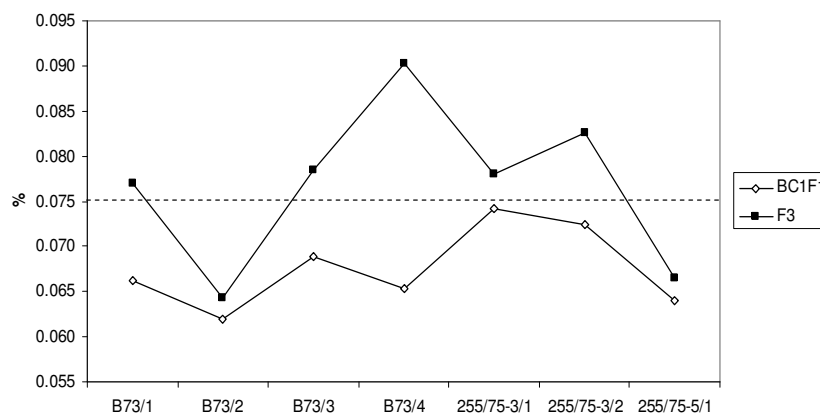
Table 1 Tryptophan content (%) in whole grains of BC1F1 and F3 genotypes of crosses between standard and QPM germplasm

Standard inbred line	QPM parental line	Tryptophan content (%)			
		BC1F1	Average	F3	Average
ZPL175	CML144	0.055	0.056	-	-
	CML159	0.053		-	
	CML161	0.057		-	
	Mat-pro	0.059		-	
B73	CML144	0.066	0.066	0.077*	0.077
	CML159	0.062		0.064	
	CML161	0.069		0.078*	
	CML165	0.065		0.090*	
	SussF	-		0.079*	
255/75-3	Mat-pro	-	0.073	0.077*	0.085
	CML144	-		0.085*	
	CML161	-		0.095*	
	CML165	0.074		0.078*	
255/75-5	SussF	0.072	0.064	0.083*	0.076
	CML144	-		0.086*	
	CML159	0.061		-	
	CML161	0.067		-	
		CML165	0.064	0.067	

\*tryptophan contents over the threshold limit (0.075)

The ears of maize F3 plants of all four crosses between standard line ZPL175 and CML144, CML159, CML161 and Mat-pro were barren, while ATC in BC1F1 genotypes of the same crosses was far below the QPM limit, i.e. 0.056 (from 0.053 to 0.059). Genotypes with 255/75-3 as a parental line showed the highest ATC in both F3 (0.085) and BC2 (0.073) compared with all the other crosses. The results of tryptophan content analysis for each genotype are given in Table 1.

Out of 18 crosses between standard and QPM lines, only seven survived in both BC1F1 and F3 generations, while the other 11 gave barren ears in one of the two generations (Table 1). It is obvious that tryptophan content is higher in F3 compared to BC1F1 genotypes of the same cross (Graphic 4). Only two genotypes derived from B73 x CML159 and 255/75-5 x CML165 had tryptophan content below the QPM limit in both generations. The highest difference between generations was detected in B73 x CML165 (0.025) and the lowest in 255/75-3 x CML165 (0.004).



Graphic 4. Whole grain tryptophan content (%) in BC1F1 and F3 progeny of individual crosses between standard and QPM germplasm (dashed line – tryptophan threshold level for QPM) Legend: B73/1 – B73 x CML144; B73/2 – B73 x CML159; B73/3 – B73 x CML161; B73/4 – B73 x CML165; 255/75-3/1 – 255/75-3 x CML165; 255/75-3/2 – 255/75-3 x SussF; 255/75-5/1 – 255/75-5 x CML165.

## DISCUSSION

Due to segregation of genes for endosperm hardness varying degrees of softness/hardness are expressed in the endosperm of segregating generations, i.e. varying degrees of opaqueness are observed on the light table. Light tabling is done to pick out kernels with the *o2o2* genotype by using the degree of opaqueness as an indirect measure or secondary trait. It is recommended to select modification score 3 in F2 generation, scores 2 and 3 in the next two generations and score 2 in the

remaining advanced generations (VIVEK *et al.*, 2008). Type 3 kernels are the best choice in early generations, as it is a compromise between the guaranteed presence of *o2o2* (a higher priority) and good modification (which can be improved in subsequent generations). Type 1 kernels are most probably *O2O2* or *O2o2*, type 5 kernels are the same as original *o2o2* mutant and type 4 kernels have low probability of obtaining good modifications in subsequent generations. That is way these kernels are discarded throughout the selection process.

The modifications in the analyzed segregating generations of different crosses between QPM, *opaque2* and standard maize germplasms were overall good. Most of the genotypes had kernel modifications in the score range from 2 to 3. Good kernel modifications were detected in over 60% of all genotypes in the three types of crosses. Even those genotypes that were scored as with poorly modified kernels had a small portion of well modified kernels which were used for further selection. Only three genotypes (two QPM x *opaque2* and one standard line x QPM) were completely discarded because they did not produce enough type 2 and type 3 kernels. Most of the genotypes with well modified kernels derived from well modified F2 and BC1 kernels (78% of all analyzed genotypes), but in 14% of the genotypes modifications were downgraded (data not presented). The mechanisms by which the endosperm modifiers change the grain structure from chalky to vitreous is not clearly understood, neither have the genes been characterized in terms of their number, chromosomal location, nor expression levels in different genetic background (PRASSANA *et al.*, 2001; SOFI *et al.*, 2009). Information available so far strongly suggest the occurrence of two major loci on chromosome 7 influencing endosperm modification from the opaque to vitreous phenotype (LOPES *et al.*, 1995), with several minor factors possibly fine-tuning the process (PRASSANA *et al.*, 2001).

Lysine and tryptophan concentrations in maize kernels of QPM are highly correlated (NURIT *et al.*, 2009). A 3:1 ratio of lysine to tryptophan has been reported in normal and quality protein maize (BJARNSON *et al.*, 1992; VIVEK *et al.*, 2008). Therefore, in a QPM breeding program only tryptophan content is monitored, due to simpler and faster determination method. The whole grain tryptophan content in F3 and BC1F1 genotypes of crosses between QPM and *opaque2* germplasm was at level of quality protein maize tryptophan content, with a few exceptions. All BC1F1 genotypes of standard lines x QPM had tryptophan content in the range of normal maize, while majority of F3 genotypes had tryptophan content at level of QPM. All the genotypes that had low tryptophan content will be discarded from further breeding.

The progeny (with increased tryptophan levels) of QPM and *opaque2* crosses had significantly higher tryptophan content compared to the progeny of crosses between standard and QPM lines – 0.098 to 0.114 and 0.080, respectively. This was expected, as QPM and *opaque2* parental lines have homozygous recessive *o2o2 opaque2* gene and thus increased tryptophan content, while standard lines are homozygous dominant *O2O2* and thus poor in tryptophan.

Besides tryptophan content, quality index (tryptophan % in the protein) is also essential for proclaiming maize a QPM genotype. Although the limits for both



tryptophan and quality index are set (VIVEK *et al.*, 2008), some QPM lines are to a certain extent below them. OKELLO *et al.* (2006) tested six QPM lines and six normal inbred lines for endosperm tryptophan content, agronomic performances and disease resistance. In three QPM lines analyzed quality index was from 0.060 to 0.068 (limit > 0.07), although their tryptophan content was high. However, these quality index values were higher compared to majority of normal lines (five lines below 0.05 and one 0.066). Quality index was not performed on BC1F1 and F3 genotypes in our work due to time limit considering sowing and the fact that either F3 or F4 generation are recommended as the first generation to determine protein quality (VIVEK *et al.*, 2008). Tryptophan content was determined with the aim to discard genotypes not only with inadequate kernel modifications but also with low tryptophan values.

Having in mind the number of different genes that are needed to produce a quality protein genotype (*opaque2* and different modifier genes), it can be said that from a simple genetic system in *o2* QPM has evolved by combination of various genetic systems, behaving to be handled like classical quantitative trait (SOFI *et al.*, 2009).

#### CONCLUSION

In the majority of different F3 and BC1F1 genotypes of QPM, *opaque2* and standard lines crosses kernel modifications and tryptophan content were in the categories which enable further breeding for quality protein maize. The only exception was BC1F1 of standard lines and QPM crosses, with no increased tryptophan content in any of the analyzed genotypes, but the results in F3 were promising for improving several standard lines for protein quality. However, it is necessary to determine agronomical characteristics that play an important role in developing desirable genotypes and this will be performed in the next breeding generation.

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## MODIFIKACIJA ZRNA I SADRŽAJ TRIPTOFANA U SEGREGIRAJUĆIM GENERACIJAMA KUKURUZA VISOKOG KVALITETA PROTEINA

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### I z v o d

Iako recesivna *opaque2* (*o2*) mutacija značajno povećava sadržaj lizina i triptofana u zrnu kukuruza, inkorporacija *opaque2* u visoko prinodne genotipove nije uspela, zbog brojnih problema koji su se javili kao posledica mekog endosperma. Kukuruz visokog kvaliteta proteina (QPM) je *opaque2* kukuruz sa poboljšanom tvrdoćom zrna i dobrim agronomskim performansama, stvoren prvenstveno od tropske i subtropke germplazme. U Institutu za kukuruz je u toku projekat koji za cilj ima poboljšanje sopstvenih *opaque2* linija i prevođenje standardnih linija u QPM kukuruz. U ovom radu predstavljamo rezultate analiza modifikacije zrna i sadržaja triptofana u F3 i BC1F1 generacijama ukrštanja QPM x *opaque2*, *opaque2* x QPM i standardne linije x QPM. Većina genotipova je imala zrno tipa 2 i tipa3 (dobre modifikacije). Sadržaj triptofana u celom zrnu genotipova ukrštanja QPM i *opaque2* linija je bio na nivou karakterističnom za QPM, sa nekoliko izuzetaka. Sadržaj triptofana u potomstvu ukrštanja standardne linije x QPM je bio povećan kod većine F3 genotipova. Potomstvo (sa povećanim sadržajem triptofana) ukrštanja QPM i *opaque2* linija je imalo znatno veći sadržaj ove aminokiseline u odnosu na potomstvo ukrštanja standardne linije x QPM - od 0.098 do 0.114 prema 0.080. Genotipovi sa lošim modifikacijama zrna i/ili niskim sadržajem triptofana će biti eliminisani iz daljeg procesa selekcije.

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