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GENETIC VARIABILITY OF ALBUMIN-GLOBULIN CONTENT, AND LIPOXYGENASE, PEROXIDASE ACTIVITIES AMONG BREAD AND DURUM WHEAT GENOTYPES

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The classical Osborne wheat proteins fraction (albumin-globulin), as well as several polypeptides from the non-gluten protein fraction using SDS-PAGE analyses were determined in the grain of five bread (*T. aestivum* L.) and five durum wheat (*T. durum* Desf.) genotypes. In addition, the activity rate of lipoxygenase (LOX) and peroxidase (POD) enzymes implicated in the antioxidant metabolism was determined.

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Albumins and globulins in wheat grains were characterized by rich protein pattern – the number of bands varied from 19 to 23 and they were defined by molecular weight 76.4–12.4 kDa. The great differences between bread and durum wheat polypeptide contents were found. Result pointed out that polypeptides with molecular weight of 65.6, 43.5 and 32.9 kDa could be used to differentiate the bread from durum wheat.

Significant differences in the LOX and POD activity between and within two wheat species were detected. Present study showed in durum wheat a lower activities of LOX and POD enzymes for about 45 and 22%, respectively, than in bread wheat.

Our results could be useful for plant breeders in screening and selecting of better raw materials with high protein quality for the flour, breadmaking and pasta industry.

Key words: bread and durum wheat, globulin fraction, lipoxygenase, peroxidase

.INTRODUCTION

Wheat is one of the primary grains consumed by humans and is grown around the world in diverse environments. Many studies have indicated that wheat quality was affected significantly by genotype, environment, and genotype-by-environment interaction (DODIG *et al.,* 2007; ZHU and KHAN, 2001). Wheat flour is functional in a whole range of applications such as the production of bread, pasta, noodles and cookies. Many flour constituents play a role in wheat flour functionality. However, it is well established that the properties of its proteins are crucial.

Mature wheat grains contain 8% to 20% proteins. Total protein content, as well as protein quality, as measured by SDS sedimentation volumes and size-exclusion HPLC, are highly influenced by environmental and genetical factors. Wheat proteins show high complexity and different interactions with each other, thus making them difficult to characterize. The molecular weight (MW) of protein generally ranges from thousands to millions, with those of wheat proteins being from 30,000 to more than 10 million Daltons (Da) (WIESER, 2007). Usually, wheat proteins are classified according to their solubility. Following the sequential Osborne extraction procedure, highly heterogenus group of nongluten proteins (albumins and globulins) and gluten proteins are isolated. The gluten proteins consist of monomeric gliadins and polymeric glutenins. Glutenins and gliadins are recognized as the major wheat storage proteins, constituting about 75-85% of the total grain proteins with a ratio of about 1 : 1 in common or bread wheat (BELDEROK *et al.,* 2000). Heat stress during grain filling caused the decrease of glutenin to gliadin ratio because gliadin synthesis continued during heat stress while there was a greatly decreased synthesis of glutenin proteins (BLUMENTAL *et al.,* 1998). The nongluten proteins (albumins and globulins) of wheat endosperm represent 20 to 25% of total grain protein (BELDEROK *et al.,* 2000; MERLINO *et al.,* 2009) and majority of them are monomeric. Their MWs is mostly lower than 25,000 Da, although a significant proportion has a MW between 60,000 and 70,000 Da (SINGH *et al.,* 2001). Futhermore, bouth albumins and

globulins contein proteins that occur as polymers stabilized by inter-chain disulfide bonds (KAWAGOE *et al.,* 2005). Nutritionally, the albumins and globulins have a very good amino acid balance. They are relatively high in lysine, tryptophan and methionine. Many of these proteins are enzymes involved in metabolic activity. Also, polymeric globulins (triticins) are strongly related with 11S legume-type globulins. Such globulins form a minor group (approximately 5% of total protein) of wheat storage proteins (YADAV and SINGH, 2011). However, several other proteins have unknown functions and are not well characterized. Some nongluten proteins, particularly those belonging to a family of trypsin and α-amylase inhibitor, are also implicated in plant defense (SHEWRY and LUCAS, 1997), but the role of α-amylase and trypsin inhibitors as wheat allergens in baker's asthma has been demonstrated (TATHAM and SHEWRY, 2008). Most of the physiologically active nongluten proteins also influence the processing and rheological properties of wheat flour. In recent years, the benefits of the use of amylases, xylanases, lipoxygenase (LOX), pentosanase, glucoseoxidase, peroxidase (POD) has stimulated further interest in the bread-making industry (JIMÉNEZ and MARTÍNEZ-ANAYA, 2001; TOYOSAKI, 2007). From the food quality point of view, the potential effects of the products formed during enzymatic reactions are much more important than the reaction itself. The reaction of LOX on its substrate generates highly reactive compounds that are initiators of a cascade reaction in which components may be affected secondary, resulting in direct losses of nutritive value, alterations of organoleptic properties and color. Loss of color observed during pasta processing is due to the LOX-linoleic system, which is responsible for carotenoid oxidation (TRONO et al., 1999; SERPEN and GÖKMEN, 2007). Although other enzymes such as peroxidases and polyphenoloxidases can contribute to semolina bleaching, a major role appears to be played by LOX (BORRELLI *et al.,* 2003).

The goals of this study are as follows: i) to determine different protein components of bread and durum wheat albumins-globilins using SDS-PAGE analyses; ii) to determine the activity of lipoxygenase and peroxidase as important nongluten proteins. A more detailed knowledge of the variability of proteins accumulation among new varieties, could facilitate ongoing efforts to improve both quantity and quality of wheat protein and could influence the selection of better raw materials for the flour and breadmaking industry.

MATERIALS AND METHODS

Wheat samples

The experimental material consisted of four bread (*Triticum aestivum* L.) and four durum (*Triticum durum* Desf.) wheat genotypes (breeding lines and cultivars) recently developed at the Maize Research Institute Zemun Polje (MRIZP), Serbia. The genotypes were chosen on the basis of their differences in agronomic traits such as yield and its components. In addition, one bread (recently wide spread in Serbia) and one durum (good pasta quality) foreign cultivar was used for comparison. Their names, pedigrees, origin and growth type are given in Table 1. Grain samples of bread and durum wheat were collected from plants grown in a

field-trial at the MRIZP in 2009-2010 growing season. The experiment was laid out in the randomized complete block design (RCBD) with two replications. Each plot consisted of eight 5-m rows at 12.5 cm spacing (machine sowing). Standard agronomic practices were used to provide adequate nutrition and to keep the plots free of diseases.

For the analysis of both wheat species, the wholemeal (particle size<500 μm) was obtained by grounding wheat grains on a Cyclotec 1093 lab mill (FOSS Tecator, Sweden).

Table 1. Name, pedigree, growth type and origin of bread and durum genotypes; country code from the UN website

Varieties	Parents (Origin)	Country	Growth type
Bread wheat			
ZP 87/I	L-99 (SRB) x Pobeda (SRB)	SRB	winter
ZP Zemunska rosa	Skopljanka (MKD) x Proteinka (SRB)	SRB	winter
ZP 224	L-4 (SRB) x Dulus/Metso (CIMMYT)	SRB	facultative
ZP Zlatna	Jasenica (SRB) x Rodna (SRB)	SRB	winter
Apache		FRA	winter
Durum wheat			
ZP 34/I	SOD 55 (SVK) x Korifla (ICARDA)	SRB	facultative
ZP 10/I	Windur (DEU) x Rodur (ROU)	SRB	winter
ZP DSP/01	Windur (DEU) x SOD 64 (SVK)	SRB	winter
ZP 7858	Mina (MKD) x Mexicali 75 (CIMMYT)	SRB	facultative
Varano		ITA	facultative

ICARDA = International Center for Agricultural Research in the Dry Areas (SYR)
CIMMYT = International Maize and Wheat Improvement Centre (MEX)

Analytical procedures

Extraction of albumin-globulin fraction

Defatted wheat flour (0.5 g) was sequentially extracted by the Osborne procedure described by ŽILIĆ *et al. (*2011). The flour was extracted with an aqueous solution of 0.5 M NaCl (10 ml). Extraction was done by repeated stirring three times for 30 min at 4^{o}C, followed by centrifugation at 20 000 g for 15 min. All supernatants (globulin (Glob) extracts) were transferred to the volumetric flask and 0.5 M NaCl was added to 50 mL. The centrifugate was vortexed with deionized water (10 ml) for 1 min, than set for 5 min, centrifugated, and the supernate discarded.

Protein content was calculated, in each fraction, from the nitrogen content determined by micro Kjeldahl method, using 5.7 as the conversion factor. The results are given as percentage of dry matter (d.m.), as well as percentage of total protein (protein solubility index-PSI).

SDS-PAGE gel electrophoresis

Extractable protein composition of the defatted samples was detected by the sodium dodecyl sulfate - polyacrilamide gel electrophoresis (SDS-PAGE) performed according to FLING and GREGERSON (1986), on 12.5% separating gels and 5% stacking gels in vertikal electrophoretic unit (LKB, Sweden). Albumin-globulin was extracted by the Osborne procedure described by ŽILIĆ *et al.* (2011). Prior to the electrophoresis, extractable proteins have been diluted in the ratio 1 : 2 (v/v) with the sample buffer (0.055 M Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, 4.3% (v/v) β-mercaptoethanol, 0.0025% (w/v) bromophenol blue), heated at 90˚C for 5 min and cooled at the room temperature. Twenty five µl of dilluted fraction of albumin-globulin fraction were loaded per well. Gels were run at 50 mA for five hours, fixed and stained with 0.23% (w/v) Coomassie Blue R-250 dissolved in 3.9% (w/v) trichloroacetic acid (TCA), 6% (v/v) acetic acid and 17% (v/v) methanol for 45 min. Destaining was performed with 8% acetic acid and 18% (v/v) ethanol. Molecular weights of the polypeptides were estimated by using low molecular weight standards (Amercham Biosciences, Sweden). The protein bands on the destaned gel were quantitated using SigmaGel sotware version 1.1 (Jandal, San Rafael, CA). The concentration of wheat proteins and their ration were calculated from the sum of the total area of their subunits and expressed as percentage of total extractable proteins. To investigate varietals effect, electrophoresis was performed in triplicate. Namely, three aliquots of the same sample were analyzed at the same time. Two gels were run simultaneously in the same electrophoretic cell.

Lipoxygenase activity

The LOX (EC 1.13.11.12) activity was determined in the crude wholemeal homogenate prepared by shaking the sample with one volume of 0.2 M sodium phosphate buffer (pH 7.5) at 4^{o}C for 120 min. The supernatant obtained by centrifugation at 20 000 g for 15 min, was used to measure the LOX activity. The assay mixture consisted of 50 mM linoleic acid in 0.2 M sodium phosphate buffer, pH 6.5, and an aliquot of the sample. The initial rate of the absorbance changes at 234 nm (ε=2.5 x 10^4 M^{-1} cm^{-1}) was recorded. The LOX activity was expressed in µmol of conjugated diene formed per minute and g d.m. (LEENHARDT *et al.,* 2006a).

Peroxidase activity

To determine the POD (EC 1.11.1.7) activity, wholemeal (0.5 g) was extracted in 10 ml of 0.1 M K-phosphate buffer, pH 7.6 at 4^{o}C with constant stirring for 1 hour. After centrifugation at 20 000 g for 15 min the obtained supernatant was used in the POD assay with ferulic acid as a hydrogen donor. The initial rate of the

absorbance changes at 286 nm (ε=1.68 x 10^4 M^{-1} cm^{-1}) was used for the calculation of the POD activity (HADŽI-TAŠKOVIĆ ŠUKALOVIĆ *et al.,* 2003).

Statistical analysis

All chemical analyses were performed in three replicates and the results were statistically analysed. Significant differences between genotype means were determined by the Fisher´s least significant differences (LSD) test, after the analysis of variance (ANOVA) for trials set up according to the RCB design. A t-test was performed to test the significance of differences between the species means. Differences with $P<0.05$ were considered significant in both tests. The coefficient of variation (CV) was determined for each trait.

RESULTS

Data in Table 2 indicate that the content of total protein was significantly higher ($P < 0.05$) in durum (ranged from 11.04 to 12.40) than bread (ranged from 9.26 to 12.64) wheat genotypes. The mean content did not vary much among durum wheat genotypes (5.71%), but relatively a high variation was found among bread wheat genotypes (13.54%). Because grains were collected from plants grown under the equal conditions in a field-trial at the same location during the same growing season, the influence of environmental factors could be ignored. However, it should be noted that rainfalls from anthesis to maturity in the season of trial (2009-2010) probably caused overall reduction in protein content. According to our previously results, the content of total protein in bread and durum wheat genotypes grown in a field-trial in 2008-2009 growing season, ranged from 10.87 to 13.04% and 11.46 to 16.53% of d.m., respectively (ŽILIĆ *et al.,* 2010). This results could lead to the conclusion that higher average temperatures and rainless in grain filling showed effect on protein content increasing.

The average values of bread and durum wheat samples for the PSI of albumin-globulin soluble fraction were 38.45 and 38.63%, respectively. No significant difference in the mean albumin-globulin was observed between bread and durum wheat. In the present study, albumin-globulin in grain of bread wheat ranged from 34.73% (ZP Zemunska rosa) to 41.79% (Apache) of total proteins. According to STEHNO *et al.* (2008) and ABDELRAHMAN *et al.* (2004), albumin-globulin constitute from 22.29 to 30.81% and 14.25 to 33.46% of the total grain proteins in cultivars grown in Czech Republic and Sudan, respectively. Among durum wheat genotypes, content of albumin-globulin was the lowest in grain of ZP 10/I (35.52% of total proteins) and the highest in grain of ZP DSP/01 (43.75% of total proteins). The mean albumin-globulin did not vary much among bread and durum wheat genotypes (6.67% and 7.86%, respectively). STEHNO *et al.* (2008) reported that strong influence of genotype was observed in proportion of albumins-globulins in total protein and content of this protein fraction in dry matter. They calculated that the greater part of the variation was due to genetic factors (66.17%).

Table 2. The content of total protein and albumin-globulin protein fraction in grains of bread and durum wheat genotypes

Varieties	Protein	Albumins+Globulins	
	(1)	(1)	(2)
Bread wheat			
ZP 87/I	9.51^{d}	3.51^{e}	36.93^{c}
Apache	9.26^{d}	3.87^{d}	41.79^{a}
ZP Zemunska rosa	12.64^{a}	4.39^{c}	34.73^{d}
ZP 224	11.76^{c}	4.62^{b}	39.28^{b}
ZP Zlatna	12.22^{b}	4.83^{a}	39.52^{b}
F test	***	***	***
CV (%)	13.54	12.11	6.67
Durum wheat			
ZP 34/I	12.15^{a}	4.79^{ab}	39.44^{b}
ZP 10/I	11.12^{b}	3.95^{d}	35.52^{d}
ZP DSP/01	11.04^{b}	4.83^{a}	43.75^{a}
Varano	12.36^{a}	4.59^{c}	37.15^{c}
ZP 7858	12.40^{a}	4.62^{bc}	37.28^{c}
F test	*	***	***
CV (%)	5.71	7.43	7.86
Mean (bread wheat)	11.08^{b}	4.24^{a}	38.45^{a}
Mean (durum wheat)	11.84^{a}	4.56^{a}	38.63^{a}

Mean of genotype and species followed by the same letter within same column are not significantly different (P<0.05); * = significant at P<0.05; ***Significant at P<0.001; CV, coefficient of variation; (1) % of dry weight; (2) % of total protein

In our study, albumins-globulins were characterized by rich protein pattern – the number of bands varied from 19 (bread wheat-ZP 244 and durum wheat ZP 34/I) to 23 (durum wheat-Varano) and they were defined by molecular weight 76.4–12.4 kDa (Figure 1). The protein pattern of albumins-globulins was divided into two relatively wide areas 69–22 kDa and 61-22 kDa in bread and durum genotypes, respectively and 18–12 kDa. The electrophoretic pattern of tested genotypes showed high similarity and their character corresponded with the detection by Czechen authors DVOŘÁČEK and ČURN (2003). The differentiating areas between bread and durum wheat genotypes were located to the three zones: the 1st with molecular weight 76–61 kDa, the 2nd with molecular weight 48–43 kDa, the 3rd with molecular weight 33–27 kDa. DUPONT *et al.* (2006) reported that the changes in protein pattern can be influenced by external factors e.g. cultivation of cultivars in conditions of low and high mineral nutrition in the soil and different temperatures in the post-anthesis period. However, ŽILIĆ *et al.* (2011) emphasized that the seed proteins can be very suitable and useful genetic markers. It is useful to point out that the durum wheat genotype Varano, which was selected in Italy, was characterized by

the presence of strong-staining bands or polypeptide chains with molecular weight of 61.9 and 60.1 kDa that were not appeared in other durum wheat genotypes selected in Serbia. Also, this polypeptide chains were absent in all Serbian bread wheat genotypes (Table 3, Figure 1). The polypeptide chain with molecular weight of 32.9 kDa which emerges in all the analyzed durum wheat genotypes, except in the grain of ZP 34/I, was absent in all bread wheat genotypes. On the other hand, we could noticed that Serbian bread wheat genotypes ZP 87/I, ZP Zemunska rosa and ZP Zlatna possessed polypeptides with molecular weight of 65.6 and 43.5 kDa that were absent in all tested durum wheat genotypes (Table 3, Figure 1). This evidence could lead to the conclusion that these bands could be used to differentiate the bread from durum wheat. The great differences between bread and durum wheat polypeptide contents were found. For example, the mean content of polypeptide with molecular weight of 69.5 kDa was 2.01 and only 0.43% of total extractable proteins in bread and durum wheat, respectively. In bread genotypes the mean content of polypeptides (MW = 52.9 and 12.4 kDa) was about 1.6 times higher than in durum wheat grains which, on the other hand, had about 1.6 times higher content of polypeptides (MW = 37.7 and 18.4 kDa) than in bread wheat genotypes (Table 3).

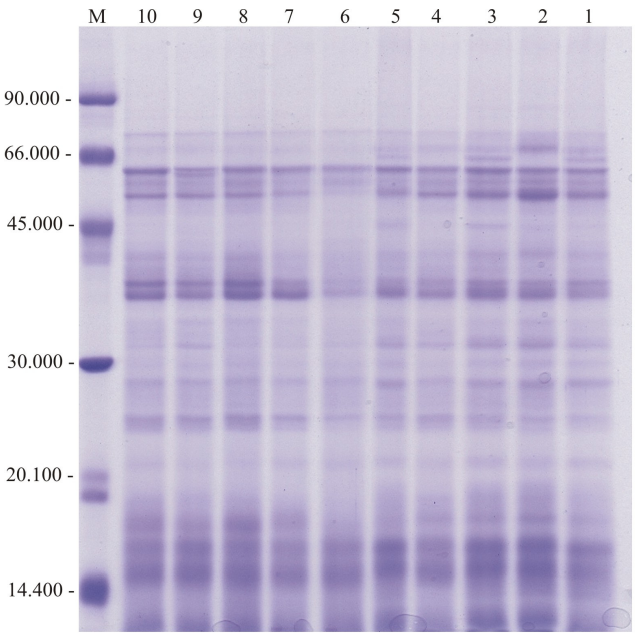

Fig. 1. SDS-PAGE patterns of albumins-globulins from bread and durum genotypes. (1-5 bread wheat), 1-ZP 87/I, 2-Apache, 3-ZP Zemunska rosa, 4-ZP 224, 5-ZP Zlatna, (6-10 durum wheat) 6-ZP 34/I, 7-10/I, 8-ZP DSP/01, 9-Varano, 10-ZP 7858. M-molecular weight standards.

Table 4. Lipoxygenase and peroxidase activity in bread and durum wheat genotypes

Varieties	LOX activity (μmol g^{-1} d.m. min^{-1})	POD activity (μmol ferulic acid g^{-1} d.m. min^{-1})
Bread wheat		
ZP 87/I	0.69^{bc}	7.73^{cd}
Apache	0.63^{c}	9.92^{b}
ZP Zemunska rosa	0.80^{ab}	10.46^{a}
ZP 224	0.89^{a}	7.61^{d}
ZP Zlatna	0.75^{b}	8.16^{c}
F test	***	***
CV (%)	12.26	18.04
Durum wheat		
ZP 34/I	0.26^{c}	4.44^{d}
ZP 10/I	0.32^{c}	6.78^{c}
ZP DSP/01	0.64^{a}	9.71^{a}
Varano	0.45^{b}	4.78^{d}
ZP 7858	0.38^{bc}	8.65^{b}
F test	***	***
CV (%)	15.16	27.25
Mean (bread wheat)	0.75^{a}	8.78^{a}
Mean (durum wheat)	0.41^{b}	6.87^{b}

Mean of genotype and species followed by the same letter within same column are not significantly different (P<0.05); ***=significant at P<0.001; CV=coefficient of variation

Although the albumin and globulin proteins are not known to play a direct role in breadmaking, as gluten proteins, manu of them, especially oxidative enzymes, may be necessary for normal baking properties (BORRELLI *et al.,* 2003). In agreement with our previously result (ŽILIĆ *et al.,* 2009), present study showed in durum wheat a lower activities of LOX and POD enzymes than in bread wheat. LEENHARDT *et al.,* (2006a) reported that LOX in einkorn grains was about 3 times less active than in durum wheat grains which were 2.5 times less active than in bread wheat varieties. The results showed that all tested bread and durum wheat genotypes contained LOX, with activity a range of 0.63-0.89 μmol g^{-1}d.m. min^{-1} and 0.26-0.64 μmol g^{-1}d.m. min^{-1}, respectively. The mean POD activity, calculated as μmoles of ferulic acid oxidised per g d.m. min^{-1}, was 8.78 and 6.87 in bread and durum wheat, respectively. FRAIGNIER et al. (2000) found large varietal differences for composition and level of POD activity. They demonstrated the presence of multiple isoforms of PODs in the durum wheat kernel, also with a tissue-specificity, suggesting for each of them a particular function. During storage or processing of wheat dough, LOX and POD could be responsible for the decline of the initial level of antioxidants. However, the activities of LOX and POD are desirable in bread making because they promote the fermentation resulting in the bread texture improvement. LEENHARDT *et al.,* (2006b)

suggested that in a perspective of bread-making the ratio between the total carotenoid concentration and the LOX activity would be a suitable criterion for wheat breeding programmes.

CONCLUSIONS

No significant difference in the mean Osborn fraction albumin-globulin was observed between bread and durum wheat. Also, the electrophoretic pattern of tested genotypes showed high similarity. However, there were a few the grain proteins which could be used as very suitable and useful genetic markers to differentiate the bread from durum wheat.

Significant differences detected in the content of albumin-globulin polypeptides, as well as in the LOX and POD activity between and within two wheat species were influenced by a genotype. Such information could be useful for plant breeders in screening and selecting of better raw materials with high protein quality for the flour, breadmaking and pasta industry.

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GENETIČKA VARIJABILNOST SADRŽAJA ALBUMINA I GLOBULINA I AKTIVNOSTI LIPOKSIGENAZE I PEROKSIDAZE IZMEĐU GENOTIPOVA HLEBNE I DURUM PŠENICE

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I z v o d

Određen je sadržaj albuminsko-globulinske frakcije, kao i polipetida koji ulaze u sastav ovih neglutenskih proteina korišćenjem SDS-PAGE analize u zrnu pet genotipova hlebne pšenice (*T. aestivum* L.) i pet genotipova durum pšenice(*T. durum* Desf.). Pored toga, određena je aktivnost oksidativnih enzima, lipoksigenaze (LOX) i peroksidaze (POD), koji su uključeni u metabolizam antioksidanasa.Albumisko-globulinska frakcija proteina zrna pšenice karakterisala se bogatom proteinskom šemom – broj traka na gelu je varirao od 19 do 23, a molekulska masa izolovanih proteina kretala se od 76.4 do 12.4 kDa. Detektovani polipeptidi u zrnu genotipova hlebne i durum pšenice razlikovali se u sadržaju. Rezultati ukazuju da bi se polipeptidi molekulskih masa 65.6, 43.5 i 32.9 kDa mogli koristiti za razlikovanje hlebne i durum pšenice. Između i unutar analiziranih vrsta pšenice detektovana je značajna razlika u aktivnosti enzima LOX i POD. Rezultati pokazuju nižu aktivnost LOX i POD u zrnu durum pšenice za 45, odnosno 22% u odnosu na hlebnu pšenicu.Dobijeni rezultati mogu doprineti selekciji osnovng materijala visokog kvaliteta proteina za upotrebu u pekarstvu i industiji testenina.

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