Cereal Research Communications 42(2), pp. 218–228 (2014)
DOI: 10.1556/CRC.2013.0070
First published online 21 February 2014

Molecular and Biochemical Analysis of Durum Wheat Genotypes to Examine Carotenoid Pigment Content and Lipoxygenase Enzyme Activity

Ö. ATEŞ SÖNMEZOĞLU[1]* and A.S. BALKAN[1,2]

[1]Department of Bioengineering, Faculty of Engineering, Karamanoğlu Mehmetbey University, Karaman, Turkey
[2]Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey

(Received 23 May 2013; accepted 24 September 2013;
Communicated by A. Goyal)

The main criterion that determines the quality of durum wheat is the degree of suitability for pasta production (pasta-processing quality). In this regard, pigment content and the quantity of oxidative enzymes of durum wheat play important roles in the quality of pasta. It is now possible to examine these features and specify their effects using recently developed genetic markers and spectrophotometric measurement techniques. In the present study, LOX enzyme activity and pigment content are determined using molecular and biochemical scanning. According to the obtained results, Gediz-75, Gdem-12, Line-19, Zenit, Line-7 and Line-20 were determined as the most suitable lines or varieties for the production of quality pasta with regard to LOX enzyme activity. As for pigment content, Kyle, Zenit, Gdem-12, Gdem-2, TMB-1 and TMB-3 showed the highest potential for the production of yellow pasta. When pigment content and LOX enzyme activity were evaluated together, the potential of the Gediz-75, Gdem-12 and Zenit durum wheat varieties and lines to produce yellow pasta products was shown to be very high.

Keywords: durum wheat, *Triticum durum,* pigment content, LOX, SSR

Introduction

Durum wheat quality is defined based on its suitability for pasta processing. The quality of pasta products is strongly correlated with the chemical and physical properties of durum wheat. Specifically, durum wheat pasta-making quality is influenced by kernel vitreousness, hectoliter weight, protein content and quality, milling properties (semolina yield and ash content), yellow pigment content and lipoxygenase (LOX), peroxidase (POD) and polyphenol oxidase (PPO) activities (Borrelli et al. 2000; Troccoli et al. 2000). Of these quality-associated parameters, protein content and quality, yellow pigment content and the activities of oxidative enzymes that adversely affect the bright yellow color of pasta

* Corresponding author; E-mail: ozlemsonmezoglu@kmu.edu.tr; Phone: +90 (338) 226 2000;
Fax: +90 (338) 226 2116

0133-3720/$20.00 © 2013 Akadémiai Kiadó, Budapest

products are of vital importance, as they overwhelmingly determine the so-called *al dente* cooking characteristics and color of pasta products.

A bright yellow color is a desired property in pasta products, making the color of pasta one of the most important quality parameters for the pasta industry (Troccoli et al. 2000). The color of pasta is affected by grain pigment content, oxidative enzymes and pasta-processing conditions.

The most distinctive pigments in the color of pasta are carotenoids and flavonoids. However, these pigments can be easily oxidized, leading to a loss of color or the so-called bleaching of pasta products. Of the oxidative enzymes, LOX most often affects the oxidative bleaching of yellow pigments in durum wheat products. Thus, wheat genotypes that are high in yellow pigments but low in oxidative enzymes are preferred for the production of pasta that has a bright yellow color (Clarke et al. 1998; Borrelli et al. 1999; Troccoli et al. 2000; Morris 2004; Aalami et al. 2007; Sakin et al. 2011). Indeed, the search for new genetic resources has been underway across the world in order to develop high-quality durum wheat genotypes.

Turkey has rich genetic resources and suitable ecological regions for durum wheat production. However, limited research has been conducted on the yellow pigments and oxidative enzymes of the durum wheat genotypes grown in Turkey (Pekin and Çakmaklı 1987; Coşkun and Ercan 2003; Sakin et al. 2011).

Further, the pasta-processing quality of durum wheat cultivars must be improved using modern breeding methods without adversely affecting grain yields (Yıldırım et al. 2013). For this purpose, existing varieties must first be investigated and screened in terms of their carotenoid pigment content and LOX enzyme activity. These features and their effects can now be examined using recently developed genetic markers and spectrophotometric measurement techniques. Thus, the determination of the most suitable cultivars for pigment content and LOX enzyme activity using both spectrophotometric and molecular genetic analysis, is the objective. For this purpose, 15 durum wheat advanced lines with different parents and seven registered varieties were used. In all genotypes, pigment content and LOX enzyme activity were determined using both biochemical and molecular analysis.

Materials and Methods

Plant materials

In this study, we used 22 durum wheat genotypes (four advanced breeding lines, 11 advanced experimental lines and seven registered varieties) (Table 1). We used molecular scanning based on DNA markers and biochemical scanning based on spectrophotometric measuring.

Molecular analysis

DNA isolation and PCR amplification

DNA was extracted from the young leaves of each plant according to the method of Doyle and Doyle (1990). DNA was screened using SSR and SCAR markers linked to the pig-

Table 1. Durum wheat genotypes used in the study

Nr	Genotype	Source / Breeding institution
1	TMB-1 (Sarıçanak//Kyle)	Advanced breeding line – TÜBİTAK- 107O004
2	TMB-2 (Salihli//Kyle)	Advanced breeding line – TÜBİTAK- 107O004
3	TMB-3 (Kızıltan//Kyle)	Advanced breeding line – TÜBİTAK- 107O004
4	TMB-4 (Selçuklu//Kyle)	Advanced breeding line – TÜBİTAK- 107O004
5	Gdem-2	Mutant line – Gaziosmanpasa University
6	Gdem-2-1	Mutant line – Gaziosmanpasa University
7	Gdem-12	Mutant line – Gaziosmanpasa University
8	Line-1 (Mrb3/Albit-1)	Advanced experimental line – ICARDA
9	Line-4 (Aghrass-2)	Advanced experimental line – ICARDA
10	Line-5 (Terbol97-1)	Advanced experimental line – ICARDA
11	Line-7 (Zna-1//Dra2/Bcr)	Advanced experimental line – ICARDA
12	Line-11 (Lagamarb-1)	Advanced experimental line – ICARDA
13	Line-19 (Gby/4/Quadalete//Erp/Mal/3/Unk)	Advanced experimental line – ICARDA
14	Line-20 (Stj3/4/Stn//Hui/Sorno/3/Yav/Fg//Roh)	Advanced experimental line – ICARDA
15	Line-24 (Rutucha-1)	Advanced experimental line – ICARDA
16	Sarıçanak-98	Registered variety – Southeast Agric. Res. Inst.
17	Salihli-92	Registered variety – Ege Agric. Res. Inst.
18	Selçuklu-97	Registered variety – Field Crops Cent. Res. Inst.
19	Kızıltan-91	Registered variety – Field Crops Cent. Res. Inst.
20	Kyle	Semiarid Prairie Agricultural Research Center
21	Zenit	Registered variety – Southeast Agric. Res. Inst.
22	Gediz-75	Registered variety – Southeast Agric. Res. Inst.

Table 2. SSR markers linked to the LOX activity

SSR marker	Primer sequence (5' – 3')	Source
Xgwm 251-4B	Forward – CAACTGGTTGCTACACAAGCA	Geng et al. 2010
	Reverse – GGGATGTCTGTTCCATCTTAG	
Xwmc 312-1A	Forward – TGTGCCCGCTGGTGCGAAG	Somers et al. 2004
	Reverse – CCGACGCAGGTGAGCGAAG	
Xwmc 692-4B	Forward – TTATCTTGATCCGAGCGA	Somers et al. 2004
	Reverse – ATGTGATTAGTCCTAAGGTCTCTCT	
Xwmc 93-1A	Forward – ACAACTTGCTGCAAAGTTGACG	Somers et al. 2004
	Reverse – CCAACTGAGCTGAGCAACGAAT	

ment as well as SSR markers linked to LOX enzyme activity. For this purpose, we used nine primers developed by different researchers (Röder et al. 1998; Somers et al. 2004; Patil et al. 2008; Geng et al. 2010) (Tables 2 and 3).

PCR amplification

PCRs were performed under the conditions given in the source articles for each primer with some modifications. The PCR reaction volume was 40 µl. The PCR reactions contained 50–100 ng of genomic DNA, 250 nM each of the primers, 0.2 mM each of the nucleotides, 1.5 mM $MgCl_2$, 10× PCR buffer and 0.5 units of *Taq* DNA polymerase (Promega).

Table 3. SSR and SCAR markers linked to the pigment

SSR and SCAR marker	Primer sequence (5' – 3')	Source
Xgwm 344-7B	Forward – CAAGGAAATAGGCGGTAACT	Röder et al. 1998
	Reverse – ATTTGAGTCTGAAGTTTGCA	
Xgwm 63-7A	Forward – TCGACCTGATCGCCCCTA	Röder et al. 1998
	Reverse – CGCCCTGGGTGATGAATAGT	
Xgwm 46-7B	Forward – GCACGTGAATGGATTGGAC	Röder et al. 1998
	Reverse – TGACCCAATAGTGGTGGTCA	
Xgwm 408-5B	Forward – TCGATTTATTTGGGCCACTG	Röder et al. 1998
	Reverse – GTATAATTCGTTCACAGCACGC	
Xscar 3362-7A	Forward – TTGGCTTATTCCAATGCACA	Patil et al. 2008
	Reverse – TGTAAGGGCAACTCCCACAT	

PCRs were run on a Bioneer (MyGenie 96) thermocycler as follows: an initial denaturation step of 3 min at 94°C was followed by 32 cycles of 1 min at 94°C, 1 min at 50–60°C (different annealing temperature of primers), and 1 min at 72°C. We concluded with a final extension step for 5 min at 72°C. PCR products were run on 3% MetaPhor^{TM} agarose gels with 1% TBE buffer. Electrophoresis was applied at a 90-watt constant power for 4–5 hours.

Biochemical analysis

Determination of LOX enzyme activity

For the determination of LOX enzyme activity, an extract was prepared according to the descriptions of Rani et al. (2001) and Aalami et al. (2007) with minor modifications. LOX was extracted by mixing 1 g of wholewheat grain with 10 ml of 50 mM sodium phosphate buffer (pH 7.5). The mixture was placed at 4°C for 1 h, stirred four times and then centrifuged (Eppendorf model 5417R, Germany) at 5,000 *g* for 15 min at 4°C. The supernatant containing LOX was used for the subsequent assay of LOX enzyme activity. The extract was stored in an ice-water bath and used the same day. Linoleic acid substrate (7.5 mM) was prepared as described by Shiiba et al. (1991) with minor modifications. Substrate solution was prepared as follows: 0.25 ml of Tween 20 was dissolved in 2.8 ml of 0.2 M sodium acetate buffer (pH 5.5) and 0.1 ml of linoleic acid was added into a 0.1 ml extract. Then, the mixture was stirred rapidly and LOX enzyme activity was determined by measuring the conjugated diene absorption at 234 nm with a UV-visible spectrophotometer for 2 min. according to the description of Aalami et al. (2007). For each genotype, the assay was repeated three times. A unit of LOX enzyme activity (EU) was calculated as the change in absorbance at 234 nm per minute per gram of whole wheat grain under assay conditions.

Determination of pigment content

Carotenoid pigment content was extracted using water-saturated n-butyl alcohol from milled samples and absorption values were determined by spectrophotometer (435.8 nm).

Carotenoid pigment content analysis through the pigment extraction method was carried out according to the modified AACC 14-50 method (AACC 2000).

Pigment content was determined on 1 g of semolina extracted after 16 h with 5 ml of water-saturated n-butyl alcohol. The mixture was centrifuged (Sigma 2-16 KC, Germany) at 5,000 *g* for 10 min. Pigment content was then calculated in absorbance at 435.8 nm (Pigment Content (mg/kg) = $\text{Absorbance}_{435,8} \times 30.1$). For each genotype, the assay was repeated three times.

Statistical analysis

Analysis of variance was performed using SPSS software (version 15.0), and the Duncan test was used to compare the averages. Polymorphisms among amplified PCR bands were determined using Vilber Lourmat's Bio 1D (version 11.04). Comparisons of genotypes and genetic relationships between genotypes were estimated by Numerical Taxonomy and Multivariate Analysis System software (NTSYSpc, version 2.11). The program was also used to obtain dendrograms (Figures 2 and 3) showing the genetic distances between wheat genotypes.

Results

Molecular scanning

In detail, DNA was screened using the following SSR and SCAR markers linked to the pigment (Xgwm 344, Xgwm 63, Xgwm 46, Xgwm 408, Xscar 3362) as well as SSR markers linked to LOX enzyme (Xgwm 251, Xwmc 312, Xwmc 692, Xwmc 93) (Fig. 1).

Dendrograms were then prepared based on molecular data on LOX enzyme activity (Fig. 2) and pigment content (Fig. 3) using this SSR and SCAR marker information.

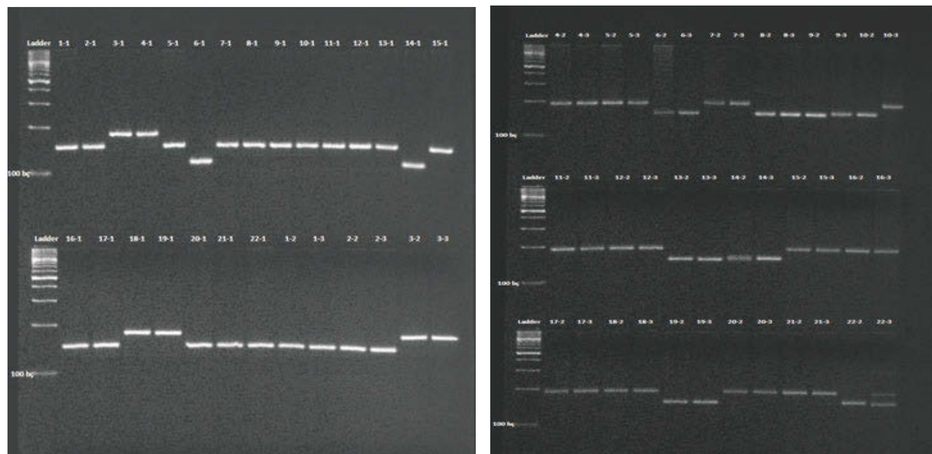

Figure 1. SSR marker profiles of durum wheat genotypes using Xwmc 93 (A) and Xgwm 408 (B). Lanes numbers depicted on the gel are listed in Table 1

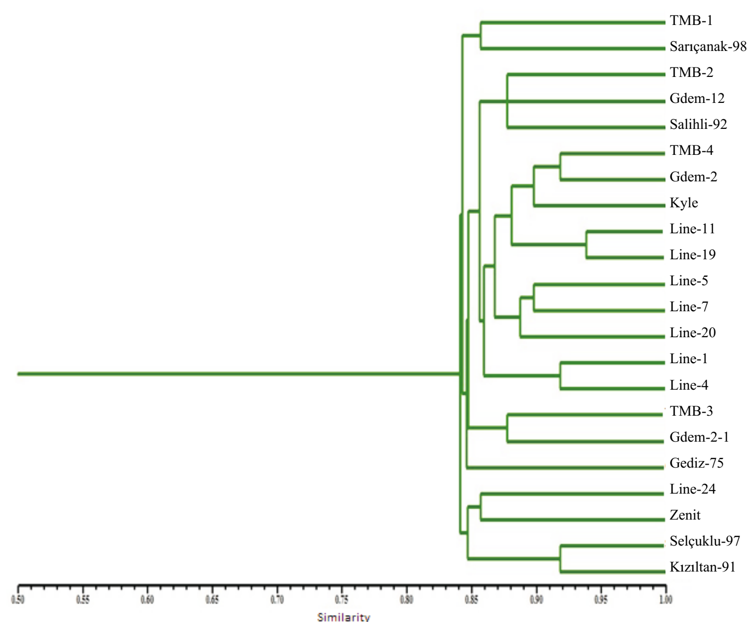

Figure 2. Dendrogram prepared based on molecular data linked to LOX enzyme activity

The durum wheat genotypes were clustered into groups and sub-groups according to the dendrogram prepared based on primers linked to LOX enzyme. The wheat genotypes formed two major groups: group 1 consisted of Kızıltan, Selçuklu, Zenit and Line-24, while group 2 included all remaining genotypes (Fig. 2).

The results from the molecular data on LOX enzyme activity showed close groups for TMB-1 and Sarıçanak-98 and for TMB-2, Gdem-12 and Salihli-92 (Fig. 2). The TMB-1 and Sarıçanak-98 and the TMB-2 and Salihli-92 varieties and lines were used in the same breeding program. Line-4, -1, -20, -7, -5, -19 and -11 were in close molecular groups. In terms of the analyzed LOX primers, Selçuklu-97 and Kızıltan-91 were found to be similar in molecular structure. Further, Gediz-75 and Zenit, which are known to have low LOX values, were included in close groups according to the molecular data (Fig. 2).

Figure 3 shows that the TMB-1 and TMB-2 and the TMB-4, Selçuklu-97 and Salihli-92 varieties and lines were in close groups according to the dendrogram prepared based on primers linked to pigment content. Additionally, Gdem-2, Gdem-12 and Kızıltan-91 were in similar groups, while Line-4, -5, -7 and -24 were found to be similar. Moreover, the Kyle and Zenit durum wheat varieties were in close molecular groups. In addition, the spectroscopic pigment measurements of the same varieties were close: 7.682 ppm and 7.719 ppm, respectively (Table 4). Finally, the TMB-1, TMB-2, TMB-3 and TMB-4 advanced breeding lines showed pigment values between 5.6 to 6.5 ppm.

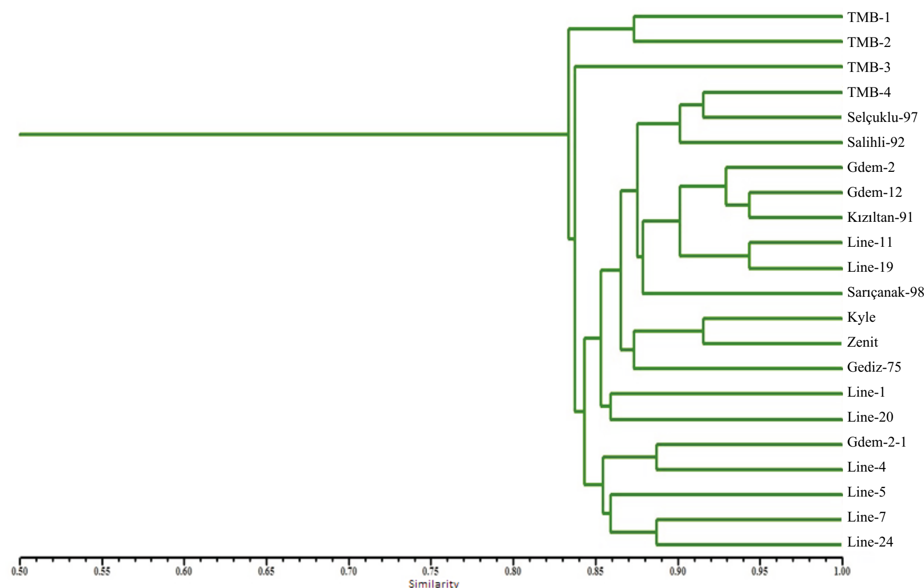

Figure 3. Dendrogram prepared based on molecular data linked to pigment content

Spectroscopic measurement scanning

The spectrophotometric measurement results obtained for LOX enzyme activity and pigment content are presented in Table 4. Whenever differences were significant ($P < 0.05$), Duncan's multiple range test was applied to separate the means. LOX enzyme activity ranged from 20.941 to 73.739 EU/g with a mean of 42.427 EU/g (Table 4).

The genotypes that showed the lowest LOX enzyme activity were Gediz-75 (20.9 EU/g), Gdem-12 (22.8 EU/g), Line-19 (24.4 EU/g), Zenit (25.9 EU/g) and Line-20 (29.7 EU/g). By contrast, TMB-4 (73.7 EU/g), TMB-3 (66.3 EU/g) and Kyle (61.6 EU/g) were the highest LOX enzyme activity genotypes (Fig. 4A).

Line-7 and Line-20 had close values based on the spectrophotometric measurements; these wheat lines were also in close groups according to the molecular screening results. Gediz-75 and Zenit, well-known low LOX enzyme activity cultivars (Coşkun and Ercan 2003; Sakin et al. 2011), contained the lowest levels of LOX enzyme activity in this study (20.941 and 25.932 EU/g, respectively).

Pigment content ranged from 3.441 to 8.644 ppm with a mean of 5.639 ppm (Table 4). The genotypes that showed the highest LOX enzyme activity were Gdem-2 (8.6 ppm), Zenit (7.7 ppm), Kyle (7.6 ppm), TMB-3 (6.5 ppm), Gdem-12 (6.3 ppm) and TMB-1 (6.0 ppm). Kızıltan-91 (3.4 ppm), Salihli-92 (4.0 ppm), Line-24 (4.1 ppm), Gdem-2-1 (4.6 ppm), Line-19 (4.6 ppm) and Line-7 (4.7 ppm) had the lowest average pigment values (Fig. 4B).

Line-4 and -5 were in the same group (a_6) according to the pigment values and also in close groups based on the molecular screening results. Line-1 and Line-20 had similar pig-

Table 4. The mean spectroscopic measurement values for the LOX enzyme and pigment

Genotype	LOX activity		Pigment amount	
	Mean (EU/g)	Statistical group	Mean (ppm)	Statistical group
TMB-1	51.334	a_9a_{10}	6.063	a_7a_8
TMB-2	50.748	a_9a_{10}	5.638	a_5a_6
TMB-3	66.375	a_{12}	6.587	a_{10}
TMB-4	73.739	a_{13}	5.868	a_6a_7
Gdem-2	51.016	a_9a_{10}	8.644	a_{12}
Gdem-2-1	48.796	a_8a_9	4.680	a_3
Gdem-12	22.758	a_1a_2	6.393	a_9a_{10}
Line-1	49.428	a_8a_9	5.651	a_5a_6
Line-4	39.165	a_6a_7	5.876	a_6a_7
Line-5	36.089	a_6	5.611	a_5a_6
Line-7	28.034	a_3a_4	4.737	a_3
Line-11	46.980	a_8	5.624	a_5a_6
Line-19	24.484	a_2	4.626	a_3
Line-20	29.764	a_4a_5	5.157	a_4
Line-24	49.524	a_8a_9	4.123	a_2
Sarıçanak-98	32.703	a_5	5.248	a_4
Salihli-92	30.832	a_4a_5	4.052	a_2
Selçuklu-97	41.427	a_7	5.141	a_4
Kızıltan-91	51.683	a_9a_{10}	3.441	a_1
Kyle	61.651	a_{11}	7.682	a_{11}
Zenit	25.932	a_2a_3	7.719	a_{11}
Gediz-75	20.941	a_1	5.500	a_5

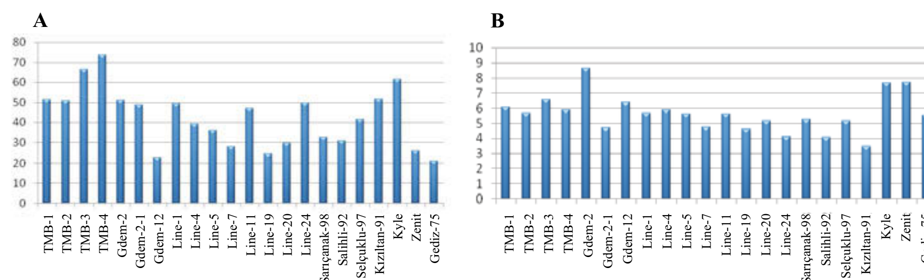

Figure 4. Graph of LOX enzyme activity (A) and pigment content (B) values

ment content (5.6 and 5.2 ppm, respectively) and were in the same group according to the molecular screening results. Similarly, TMB-4 and Selçuklu-97 as well as Kyle and Zenit were in the same groups and had close pigment values.

Discussion

The main criterion that determines the quality of durum wheat is the degree of suitability for pasta production. Pigment content and the quantity of oxidative enzymes in durum

wheat are important, as they influence the quality of pasta products (Aalami et al. 2007; Permyakova et al. 2010). LOX enzyme activity and pigment content thus play a decisive role in pasta properties (Bushuk 1998; Troccoli et al. 2000). For the production of high-quality pasta products, durum wheat is generally expected to contain low LOX enzyme activity and high yellow pigment content.

It has generally been reported that the pigment content of durum wheat must be between 4 and 8 mg kg^{-1} (Koksel et al. 2000; Troccoli et al. 2000; Borrelli et al. 2003). The results of this study thus indicate that the Kyle, Zenit, Gdem-12, Gdem-2, TMB-1 and TMB-3 genotypes have high potential for the production of bright yellow pasta products according to the statistical data presented herein. We also determined that TMB-4, Line-4, Line-1, Line-5, Line-11 and Gediz-75 have suitable values for the desired pigment content (5.5–5.9 ppm). This confirms that Gediz-75, Gdem-12, Line-19, Zenit, Line-7 and Line-20 are the most suitable genotypes for the production of quality pasta according to the statistical data on LOX enzyme activity.

In this study, the durum wheat varieties Gediz-75 and Zenit with the lines Gdem-12 and Line-19 showed the lowest LOX enzyme activity, but Gdem-2 with Kyle and Zenit had the highest level of pigment content. Gediz-75 and Zenit, well-known low LOX enzyme activity cultivars (Coşkun and Ercan 2003; Sakin et al. 2011), contained the lowest level of LOX enzyme activity in this study. Based on these results, Gediz-75, Gdem-12 and Zenit may be more suitable for pasta production.

In the present study, LOX enzyme activity and pigment content varied among the wheat genotypes. Other researchers have also reported that the durum wheat genotypes grown in Turkey exhibit large variations in pigment content and oxidative enzymes (Coşkun and Ercan, 2003; Sayaslan et al. 2012). When considered by pigment content and LOX enzyme activity, many lines and varieties have great potential to produce yellow pasta products.

In another study that investigated the genetic variability of carotenoid concentration as well as LOX and POD activities among cultivated wheat species and bread wheat varieties, it was determined that the LOX enzyme activity of wheat was negatively correlated to total carotenoid concentration (Leenhardt et al. 2006). In the present study, varieties having high pigment content also have low LOX enzyme activity.

Results of this study are shown that the varieties and lines having potential for high-quality of the final product were determined using both biochemical and molecular analysis. Methodologically, pigment content and LOX enzyme activity were determined using molecular scanning based on DNA primers and biochemical scanning based on spectrophotometric measuring. These markers can be used alone or in combination with the biochemical analysis for marker-assisted selection in wheat breeding. Similarly, molecular and biochemical scanning successfully used combined marker assisted selection (Ateş Sönmezoğlu et al. 2010). With the help of marker-assisted selection, breeding time can be reduced and the efficiency of breeding can be increased (Yıldırım et al. 2013). The selection for LOX enzyme activity can be effectively applied at an earlier generation in the breeding process (Geng et al. 2010). The results of this study confirm that molecular and biochemical scanning can be employed successfully in wheat breeding programs.

Acknowledgement

This research has been financially supported by Karamanoğlu Mehmetbey University Scientific Research Projects (BAP-04-L-11).

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