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Effects of Genotype, Growing Season and Nitrogen Level on Gluten Protein Assembly of Durum Wheat Grown under Mediterranean Conditions

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Abstract: Water deficit and high temperatures are the main environmental factors which affect both wheat yield and technological quality in the Mediterranean climate. The aim of the study was to evaluate the variation in the gluten protein assembly of four durum wheat genotypes in relation to growing seasons and different nitrogen levels. The genotypes, Marco Aurelio, Quadrato, Pietrafitta and Redidenari, were grown under three nitrogen levels (36, 90 and 120 kg ha^{-1}) during two growing seasons in Southern Italy. Significant lower yield and a higher protein concentration were observed in the year characterized by a higher temperature at the end of the crop cycle. The effect of the high temperatures on protein assembly was different for the genotypes in relation to their earliness. Based on PCA, in the warmer year, only the medium-early genotype Quadrato showed positive values along the "protein polymerization degree" factor, while the medium and medium-late genotypes, Marco Aurelio and Pietrafitta showed negative values along the "proteins assembly" factor. No clear separation along the two factors was observed for the early genotype Redidenari. The variation in gluten protein assembly observed in the four genotypes in relation to the growing season might help breeding programs to select genotypes suitable for facing the ongoing climate changes in Mediterranean area.

Keywords: durum wheat; glutenin polymers; gluten quality; high temperature; nitrogen fertilization

1. Introduction

Durum wheat (*Triticum turgidum* L., subsp. *durum* Desf.) is the most widespread cereal crop in Mediterranean countries and is grown in various climatic conditions [1].

Water deficit and high temperatures are the main environmental factors which affect both wheat yield and technological quality in the Mediterranean climate [2,3]. According to studies performed by the Intergovernmental Panel on Climate Change (IPCC), further increase in temperatures is predicted in Europe, especially in the Southern and Central parts [4,5]. In this context, the maintenance of adequate yield and quality standards is of particular interest, since the annual variability of product quality cannot be acceptable, especially for dry pasta production [6].

The wheat grain quality mainly depends on the quantity and type of gluten proteins, as well as on their aggregation/polymerization level [7,8]. In particular, gliadins, which are monomeric proteins, are mainly responsible for the viscous nature of the dough, and interact mostly via non-covalent links, while glutenin, which are polymeric proteins stabilized by disulphide bonds, determine its elasticity [9–12].

In the literature [13–22], conflicting results on the effect of high temperatures on the quality of the gluten proteins have been reported. Studies made on bread wheat suggest that when high temperatures occur in the middle of grain filling, they positively affect dough strength [13], while very high temperatures near physiological maturity can have a negative effect [14]. Ciaffi et al. [15] reported that in bread wheat, high temperatures increased the accumulation of glutenins compared to gliadins. On the contrary, O'Leary et al. [16] reported that water or thermal stress conditions throughout the grain filling period determine a delay in the synthesis of glutenins while the synthesis of gliadins is not altered. Furthermore, for common wheat, it is reported that short periods of very high temperatures can significantly reduce the proportion of SDS-insoluble polymers (UPP) [15,17], which in bread wheat (*Triticum aestivum* L.) have been positively correlated with dough viscoelasticity [7,8]. On the contrary, some authors have reported that short periods of very high temperatures can lead to an increase in the size of glutenin polymers in both soft and durum wheat [18,19]. While numerous are the studies available in the literature on the effect of high temperatures on gluten protein concentration, composition and on polymeric proteins size and distribution in common wheat [20–22], very few are the studies relative to durum wheat and to its pasta-making quality [8]. Moreover, pasta-making quality in durum wheat is mostly determined by low-molecular-weight glutenin subunits (LMW-GS), especially the B-type [23], whereas in bread wheat high molecular weight glutenin subunits (HMW-GS) play the major role in determining dough technological properties [24].

In the Mediterranean areas, after climate conditions, the nitrogen (N) availability represents the main constraint in obtaining adequate yield and quality in durum wheat [25]. Some studies on bread wheat have suggested that high doses of N tend to increase the amount of monomer proteins [26,27] and to reduce the percentage of UPP causing an increase in the extensibility of the dough [28–31]. Moreover, some authors have highlighted that the effect of nitrogen on gluten proteins composition and on polymers organization may vary according to the genotype [26,30,32]. Finally, for the same parameters, significant effect of the interaction between the high temperatures and N availability has been reported [29,33]. Malik et al. [33] highlighted that the combinations of cultivars, nitrogen and temperature were needed to explain the variation in the quantity and size distribution of the polymer proteins and their effects on the quality of the end-product. To the best of our knowledge, for durum wheat, this type of information is still lacking.

Thus, the aim of the present study was to evaluate the variation in gluten proteins quality, in terms of their capacity to assembly in a visco-elastic structure, of four durum wheat genotypes in relation to the growing season and different nitrogen levels, including a low input rate.

2. Materials and Methods

2.1. Field Trials

Four durum wheat cultivars, Marco Aurelio, Quadrato, Pietrafitta and Redidenari, that are used in an important Italian pasta supply chain, (Table 1), were grown in two rain-fed field experiments carried out at Foggia (latitude 41°46′ N and longitude 15°54′ E, 74 m a.s.l.) during two growing seasons (2016–2017 and 2017–2018, hereafter indicated as 2017 and 2018, respectively) in a clay loam soil.

Table 1. Main characteristics of the genotypes under study.

Genotype	Year of Release	Pedigree	Earliness
Pietrafitta	1999	Grazia x Isa	medium-late
Quadrato	1999	Creso x Trinakria	medium early
Marco Aurelio	2010	Orobel//Arcobaleno/Svevo	medium
Redidenari	2015	Kofa x N185	early

The main chemical and physical soil characteristics in the two experimental year, 2017 and 2018, are reported in Table 2.

Table 2. Soil physical and chemical characteristics in the two experimental years.

Soil Characteristics		2017	2018
Sand	%	21.5	25.2
Silt	%	39.8	36.2
Clay	%	38.7	38.6
pH		8.1	8.2
Organic Matter *	%	1.9	1.9
Total Nitrogen **	‰	1.3	1.3
Assimilable Phosphorus √	mg kg^{-1}	80	64
Exchangeable Potassium ◊	mg kg^{-1}	461	422
Field Capacity (−0.03 MPa)	%	37.3	33.13
Wilting Point (−1.5 MPa)	%	19.7	18.5
Bulk Density	Mgm^3	1.15	1.10

* Walkley-Black method; ** Kjeldhal method; √ Olsen method; ◊Ammonium acetate method.

The four cultivars were sown on November 17 in 2016 and November 25 in 2017, at a seeding rate of 240 kg ha^{-1}. In both years, the experiment was in a field where the previous crop was durum wheat.

Three different nitrogen levels were adopted corresponding to 36, 90 and 120 kg ha^{-1} (N36, N90 and N120, respectively). The fertilizers used were Yara Mila Supersemina (18% nitrogen) at pre-sowing fertilization and Yara Bela Sulfan (24% nitrogen) at tillering, stem elongation and inflorescence emergence fertilization.

Each year, the experiment was arranged in a split-plot design with two factors (genotype in plots and nitrogen levels in sub-plots) and three replications; each sub-plot was 20.4 m^2.

The grain harvest was carried out at physiological maturity on 13 June 2017 and on 22 June 2018. During the experimental period, the daily climatic parameters of rainfall and temperature were recorded by a weather station near the experimental area.

2.2. Yield and Technological Quality Parameters

At harvest, grain yield (t ha^{-1}) and thousand kernel weight (TKW) were determined. Moreover, grain protein content (GPC) was performed by NIR System Infratec 1241 Analyzer (Foss, Hillerod, Denmark).

Semolina flours have been obtained from kernels milled by Bona mill 4 cylinders (sieve 180 µm).

The gluten index (GI), an indicator of the gluten strength, was determined on semolina samples using the Glutomatic system according to ICC standard 155 [34].

2.3. Calculation of %UPP and Analysis of Gluten Protein Molecular Size Distribution

The percentage of Unextractable Polymeric Proteins (%UPP) was measured trough the SE-HPLC procedure according to the method reported in Tosi et al. [35] with minor modifications. The SDS-soluble fraction was obtained by adding to the semolina a solution consisting of 0.5% (w/v) SDS in 0.05 M sodium phosphate buffer, pH 6.9 to a final concentration of 10 mg/mL (0.3 g semolina on 30 mL buffer). The mixture was stirred for 30 min at room temperature and then centrifuged at 20.000 g for 20 min at 15 °C. The supernatant was filtered through 0.45 µm PVDF filters and 20 µl were injected into a Biobasic Thermo Scientific SEC-300 Columns (300 mm × 7.8 mm; flow rate: 0.7 mL/min) and run for 40 min, with an eluent consisting of 0.05 M sodium phosphate buffer pH 6.9, containing 0.08 M NaCl and 0.1 % (w/v) SDS, using the UHPLC Ultimate 3000 Thermo scientific. Detection was at 214 nm. The SDS soluble fraction profiles were divided into four areas, corresponding to HPLC fractions F1, F2, F3 and F4 (Figure S2a). The first two areas correspond to large and medium size polymers, with both being enriched in HMW-GS (mainly F1) and B-type LMW-GS (mainly F2) of glutenin. F3 corresponds to ω-gliadins and small oligomers enriched in C-type and D-type LMW-GS subunits [23], while F4 corresponds to monomeric gliadins (α-type and β-type) and non-gluten proteins [35].

The SDS-insoluble fraction was obtained from the residue of the centrifugation step. The pellet was resuspended in 30 mL of the same extraction buffer and sonicated in a probe type sonicator (SONICS Vibracell Model VCX 130 -max output power 130 W at a frequency 20 KHz) for 30 s at 45% power setting. After centrifugation at 20.000 *g* for 20 min at 15 °C, the supernatant was filtered through 0.45 µm PVDF filters and 20 µL were injected into column in the same condition described above. The SDS-insoluble fraction profile (Figure S2b) showed only one peak (F1*) containing the largest glutenin polymers, insoluble in SDS solution alone, but rendered soluble by sonication.

Samples were extracted in duplicate and two replicate separations for each extraction were performed. The proportions of each peak (%F1* and%F1–%F4) were calculated as percentages of the total areas of the two chromatograms (SDS-insoluble and SDS-soluble fractions). The amount of monomeric over polymeric proteins (mon/pol) was calculated as the ratio between the sum of F3 and F4 areas and the sum of F1*, F1 and F2 areas. %UPP was determined as the ratio between F1* area and the sum of F1 and F1* areas (*100).

2.4. Statistical Analysis

The dataset was tested according to the basic assumptions of analysis of variance (ANOVA). The normal distribution of the experimental error and the common variance of the experimental error were verified through Shapiro–Wilk and Bartlett's tests, respectively. When required, Box-Cox transformations [36] were applied prior to analysis. The ANOVA procedure was performed according to a split-plot design with three replicates. Three-way ANOVA procedure was performed considering the factors (growing season, genotype and nitrogen level) as fixed factors. The statistical significance of the difference among the means was determined using Tukey's honest significance difference post hoc test at the 5% probability level. A principal component analysis (PCA) was performed on the correlation matrix of technological and SE-HPLC parameters. We obtained Principal Components (PCs) on centered and scaled variables, through diagonalization of the correlation matrix and extraction of the associated eigenvectors and eigenvalues. Grain protein content, gluten index, and SE-HPLC parameters were set as quantitative variables and used to define PCs, while genotype, N level and growing season were used as categorical variables, not considered in the computation of PCs. The coordinates of the categorical variables were calculated in order to enhance the interpretation of data and were represented as barycenter in the Principal Component biplot. The number of factors needed to adequately describe the data was determined on the basis of the eigenvalues and of the percentage of the total variance accounted by the different factors. The results of PCA were graphically represented in two-dimensional plot, using the SigmaPlot software (Systat Software, Chicago, IL, USA). ANOVA and PCA analyses were performed using the JMP software package, version 14.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Weather Condition

The climatic data related to the two growing seasons are reported in Table 3, while the rainfall distribution and maximum and minimum daily mean temperatures of the 2017 (a) and 2018 (b) crop seasons are reported in Figure S1 (Supplementary File).

The first growing season was characterized by lower rainfall compared to the second year (about 340 mm vs. 401 mm). Moreover, in the first experimental year the rain distribution was not regular, with the most intense rainfall occurred in the second decade of January, the third decade of February, the second decade of April and the first decade of May. As for the second growing season, rainfall was observed throughout the crop cycle, especially during the grain filling period, in the first ten days of May and June. In addition to rainfall, the two years differed also for the maximum temperatures during the grain filling period showing the second year the highest values. Moreover, during 2018, more days with temperatures between 30 and 35 °C and three days with temperatures higher than 35 °C, compared to 2017, occurred.

Table 3. Climatic data related to the two growing seasons.

		2017	2018
Crop cycle duration	d	209	210
Crop cycle rainfall	mm	339.9	401.4
From seeding to heading rainfall	mm	204.2	198.6
Grain filling rainfall	mm	135.7	202.5
Crop cycle Mean T	°C	12.3	13.1
Grain filling Mean T	°C	18.3	21.7
Grain filling Mean T max	°C	25.5	29.1
30 °C < T < 35 °C	d	15	23
T > 35 °C	d	-	3

3.2. Yield and Technological Parameters

The analysis of variance (ANOVA) generally showed a significant effect of year (Y), genotype (G) and nitrogen (N) on the parameters considered (Table S1). The two growing seasons differently influenced the yield and the technological parameters considered. In the second growing season (Table 4), a significant lower yield, a thousand kernel weight and gluten index were observed with respect to the first one. On the contrary, grain protein content was higher in 2018 than in 2017. Relative to the nitrogen level (Table 4), a significant positive effect on grain yield was evident only under N90, while for protein content the highest value was observed under N120. Finally, the gluten index values decreased with N level increasing.

Table 4. Effect of the year, nitrogen level and genotype on grain yield, thousand kernel weight, grain protein content and gluten index.

Experimental Factors	Grain Yield (t ha^{-1})	Thousand Kernel Weight (g)	Grain Protein Content (%)	Gluten Index (-)
Year				
2017	6.66 a	60.91 a	14.53 b	64.44 a
2018	5.91 b	50.21 b	16.00 a	58.50 b
Nitrogen level				
N36	6.20 b	55.16 a	14.25 c	63.83 a
N90	6.36 a	55.90 a	15.33 b	62.71 a
N120	6.28 ab	55.62 a	16.23 a	57.88 b
Genotype				
Marco Aurelio	7.11 a	50.62 d	15.74 b	57.72 bc
Pietrafitta	5.75 c	64.47 a	15.29 c	56.50 c
Quadrato	6.42 b	54.56 b	14.08 d	61.39 b
Redidenari	5.85 c	52.60 c	15.97 a	70.28 a

For each experimental factor, values in column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.

Among the genotypes (Table 4), Marco Aurelio showed the highest yield value even if associated with lower thousand kernel weight. Instead, Redidenari was the genotype with the best technological quality performance showing the highest protein content and gluten index values. However, the behavior of the genotypes changed in relation to growing seasons (Table 5) and nitrogen levels adopted (Table 6). In particular, the yield decrease observed in the second year was different among the genotypes (Table 5); it was 5% and 9% for Marco Aurelio and Redidenari, and 14% and 17% for Pietrafitta and Quadrato, respectively. Moreover, Marco Aurelio in addition to presenting lower yield decrease in the second year also showed an increase in the protein content that was double compared to the other genotypes (3.1% vs. 0.4%, 1.36% and 1.07% for Pietrafitta, Quadrato and Redidenari, respectively). Finally, as for gluten index, Marco Aurelio and Redidenari showed a significant decrease in the second year, more marked for Redidenari (Table 5).

Table 5. Effect of the year x genotype interaction on grain yield, thousand kernel weight, grain protein content and gluten index.

	2017				2018			
	Marco Aurelio	**Pietrafitta**	**Quadrato**	**Redidenari**	**Marco Aurelio**	**Pietrafitta**	**Quadrato**	**Redidenari**
Grain yield (t ha^{-1})	7.29 a	6.20 c	7.02 ab	6.12 c	6.92 b	5.31 e	5.82 d	5.57 de
Thousand kernel weight (g)	54.82 c	71.21 a	59.25 b	58.39 b	46.42 e	57.74 b	49.88 d	46.80 e
Grain protein content (%)	14.19 f	15.11 d	13.40 g	15.43 c	17.29 a	15.47 c	14.76 e	16.50 b
Gluten index (-)	60.78 b	56.44 b	60.89 b	79.67 a	54.67 c	56.56 b	61.89 b	60.89 b

In each row, values followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.

Table 6. Effect of the genotype x nitrogen interaction on grain yield, thousand kernel weight, grain protein content and gluten index.

		Marco Aurelio	**Pietrafitta**	**Quadrato**	**Redidenari**
Grain yield (t ha^{-1})					
	N36	7.16 a	5.6 f	6.46 bc	5.61 ef
	N90	7.00 a	5.99 de	6.21 cd	6.25 bcd
	N120	7.17 a	5.68 ef	6.60 b	5.68 ef
Thousand kernel weight (g)					
	N36	50.18 e	64.67 a	53.80 bcd	51.99 cde
	N90	50.76 de	65.38 a	54.22 bc	53.26 bcde
	N120	50.91 de	63.37 a	55.67 b	52.53 bcde
Grain protein content (%)					
	N36	15.33 c	14.1 e	12.8 f	14.75 d
	N90	15.60 c	15.33 c	14.03 e	16.33 b
	N120	16.28 b	16.43 ab	15.40 c	16.82 a
Gluten index (-)					
	N36	57.67 bcde	56. 00 cde	67.33 ab	74.33 a
	N90	54.33 de	61.17 bcd	66.83 abc	68.50 ab
	N120	61.17 bcd	52.33 de	50. 00 e	68. 00 ab

For each parameter, values in each row and column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.

The nitrogen fertilization did not significantly affect the grain yield response in Marco Aurelio, while for both Pietrafitta and Redidenari, the highest values were observed under N90 level; for Quadrato the highest value was observed under N120 even if not significantly different from N36 (Table 6). On the contrary, for all genotypes a positive effect of the nitrogen level on grain protein content was evident with the highest values observed under N120. The effect of nitrogen fertilization on gluten index was not clear; only Quadrato showed a significant decrease under N120 level (Table 6).

3.3. Measurement of %UPP and Analysis of Gluten Protein Molecular Size Distribution

SE-HPLC was used to compare the molecular size distribution of the semolina proteins by a quantitative comparison of elution profiles.

The analysis of variance performed on the percentage of SDS-insoluble protein fraction (F1*), SDS-soluble protein fraction (F1–F4), monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein (%UPP) showed a general significant effect of the year (Y), genotype (G), nitrogen level (N) and their interactions (Table S2). A significant decrease of F1* and %UPP was observed in 2018 compared to 2017. Moreover, in 2018 a significant increment of the polymeric fraction, due to an increase of both F1 and F2 was observed. On the contrary, in the same year, a decrease of the monomeric fraction, due to a decrease of F4 was evident, determining also a lower mon/pol ratio with respect to 2017 (Table 7). As for the nitrogen levels, a general positive effect of N90 compared with N36 was observed for F1*, %UPP and for the monomeric fraction, while there have never been significant differences between N36 and N120 (Table 7). Finally, as for genotypes, Marco Aurelio showed higher values of %UPP and polymeric fraction, due to higher values of F1* and F2, and lower value of mon/pol ratio. On the contrary Redidenari and Pietrafitta showed lower values of polymeric fraction (again mainly due to lower F1* and F2 values) and higher values of monomeric fraction and mon/pol ratio (Table 7). Finally, Quadrato showed intermediate values for all the fraction considered. The behavior of the genotypes changed in relation to growing seasons (Table 8). A significant decrease of F1* in the second year was evident for Marco Aurelio and Pietrafitta, more marked for the former. As consequence also %UPP significantly decrease in 2018 for Marco Aurelio (13.7%) and Pietrafitta (4.2%). On the contrary, a significant increase of F1* and %UPP was observed in the second year for Quadrato. All genotypes showed the increase of F1 values in the second year and only Marco Aurelio and Pietrafitta the increase of F2 values. Also for the polymeric and monomeric fraction the effect of the growing season was observed only for Quadrato and Redidenari. In particular, in 2018 these two genotypes showed higher polymeric and lower monomeric fraction values than 2017. The increase in polymeric fraction was due mainly to the significant increase in 2018 of both F1* and F1 for Quadrato, and of F1 for Redidenari, while the decrease of the monomeric fraction was due mainly to the F4 decrease.

Table 7. Effect of the year, genotype and nitrogen level on SDS insoluble (F1*) and soluble protein fraction (F1–F4) separated by SE-HPLC, monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein (%UPP).

Experimental Factors	F1*	F1	F2	F3	F4	F1*+F1	Polymeric Fraction (F1*+F1+F2)	Monomeric Fraction (F3+F4)	%UPP	mon/pol
						(%)				(-)
Year										
2017	10.66 a	24.16 b	11.52 b	22.99 a	30.68 a	34.82 b	46.34 b	53.66 a	30.20 a	1.17 a
2018	9.71 b	26.99 a	12.07 a	23.24 a	27.99 b	36.70 a	48.77 a	51.23 b	26.26 b	1.06 b
Nitrogen level										
N36	10.07 b	26.22 a	11.75 a	22.59 a	29.38 a	36.29 a	48.04 a	51.96 b	27.66 b	1.09 b
N90	10.68 a	24.69 b	11.76 a	23.46 a	29.42 a	35.37 b	47.13 b	52.87 a	30.04 a	1.13 a
N120	9.80 b	25.81 a	11.88 a	23.30 a	29.21 a	35.61 ab	47.49 ab	52.51 ab	27.00 b	1.12 ab
Genotype										
Marco Aurelio	12.32 a	25.71 a	13.22 a	22.73 b	26.02 d	38.04 a	51.26 a	48.74 c	32.18 a	0.96 c
Pietrafitta	8.46 c	25.82 a	10.91 c	24.31 a	30.50 b	34.28 c	45.20 c	54.80 a	24.64 c	1.21 a
Quadrato	10.28 b	25.38 a	11.92 b	22.97 ab	29.45 c	35.66 b	47.58 b	52.42 b	28.69 b	1.11 b
Redidenari	9.68 b	25.37 a	11.12 c	22.46 b	31.37 a	35.05 bc	46.17 c	53.83 a	27.43 b	1.17 a

For each experimental factors, values in column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.

Table 8. Effect of the year x genotype interaction on SDS insoluble (F1*) and soluble protein fraction (F1–F4) separated by SE-HPLC, monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein (%UPP).

	2017				2018			
(%)	**Marco Aurelio**	**Pietrafitta**	**Quadrato**	**Redidenari**	**Marco Aurelio**	**Pietrafitta**	**Quadrato**	**Redidenari**
F1*	14.95 a	9.18 c	8.93 cd	9.56 c	9.70 c	7.74 d	11.62 b	9.80 c
F1	23.11 d	24.98 c	24.00 cd	24.53 c	28.31 a	26.67 b	26.76 b	26.21 b
F2	12.76 b	10.32 f	12.09 c	10.90 ef	13.68 a	11.50 cde	11.76 cd	11.34 de
F3	22.50 b	23.72 ab	23.27 ab	22.45 b	22.96 ab	24.89 a	22.66 ab	22.47 b
F4	26.68 d	31.79 a	31.70 a	32.56 a	25.36 e	29.21 c	27.19 d	30.19 b
F1*+F1	38.1 a	34.2 c	32.3 c	34.1 c	38 a	34.4 bc	38.4 a	36 b
Polymeric fraction (F1*+F1+F2)	50.83 a	44.49 c	45.03 c	45.00 c	51.68 a	45.9 bc	50.14 a	47.34 b
Monomeric fraction (F3+F4)	49.17 c	55.51 a	54.97 a	55.00 a	48.32 c	54.1 ab	49.86 c	52.66 b
UPP	39.0 a	26.7 cd	27.1 cd	28.0 bc	25.3 d	22.5 e	30.3 b	26.9 cd
mon/pol (-)	0.97 c	1.25 a	1.22 a	1.22 a	0.94 c	1.18 ab	0.99 c	1.12 b

Values in each row followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.

Relative to the effect of the genotype x nitrogen level interaction (Table 9), a significant effect of nitrogen level on F1* was evident for Marco Aurelio and Redidenari; in particular, for the former the F1* values increased with N level increasing, while for Redidenari the highest value was observed under N90. Both of these genotypes showed also highest %UPP values under N90. Moreover, only Redidenari showed a significant effect of the nitrogen level on the polymeric and the monomeric fraction, showing under N120 lower polymeric and higher monomeric fraction values.

Table 9. Effect of the genotype x nitrogen level interaction on SDS insoluble (F1*) and soluble protein fraction (F1–F4) separated by SE-HPLC, monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein (%UPP).

(%)		Marco Aurelio	Pietrafitta	Quadrato	Redidenari
F1*	N36	11.18 bcd	9.25 efg	10.26 cde	9.58 def
	N90	12.70 ab	8.33 fg	9.80 def	11.91 abc
	N120	13.10 a	7.80 g	10.77 cde	7.55 g
F1	N36	25.86 ab	26.51 ab	25.81 ab	26.72 a
	N90	25.07 b	25.10 b	25.17 b	23.42 c
	N120	26.21 ab	25.86 ab	25.18 b	25.98 ab
F2	N36	13.01 a	10.73 de	11.71 bc	11.54 bcd
	N90	13.45 a	11.02 cde	12.05 b	10.50 e
	N120	13.20 a	10.98 cde	12.02 b	11.32 bcde
F3	N36	23.10 abc	23.38 abc	22.97 abc	20.9 c
	N90	23.28 abc	24.10 ab	23.36 abc	23.09 abc
	N120	21.8 bc	25.45 a	22.57 abc	23.39 abc
F4	N36	26.85 e	30.13 bcd	29.26 d	31.26 ab
	N90	25.51 f	31.45 a	29.63 d	31.09 abc
	N120	25.70 ef	29.91 cd	29.46 d	31.77 a
F1*+F1	N36	37.04 abc	35.76 bcde	36.07 bc	36.29 bc
	N90	37.76 ab	33.43 f	34.96 cdef	35.32 cdef
	N120	39.31 a	33.66 def	35.95 bcd	33.53 ef
Polymeric fraction (F1*+F1+F2)	N36	50.04 ab	46.49 cd	47.77 bc	47.88 bc
	N90	51.22 a	44.46 d	47.01 cd	45.82 cd
	N120	52.51 a	44.64 d	47.97 bc	44.84 d
Monomeric fraction (F3+F4)	N36	49.95 cd	53.51 ab	52.23 bc	52.17 bc
	N90	48.78 d	55.54 a	52.99 ab	54.17 ab
	N120	47.49 d	55.36 a	52.03 bc	55.15 a
UPP	N36	30.2 bc	25.8 def	28.5 cd	26.2 def
	N90	33.8 a	24.9 efg	27.7 cde	33.7 a
	N120	32.5 ab	23.2 fg	29.9 bc	22.4 g
mon/pol (-)	N36	1.00fg	1.15 b-e	1.10 def	1.10 de
	N90	0.96 g	1.25 a	1.14 cde	1.19 abcd
	N120	0.91 g	1.24 ab	1.09 ef	1.23 abc

For each parameter, values in each row and column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's test.

3.4. PCA Analysis

A principal component analysis (PCA) was performed on the correlation matrix. The results of PCA allowed two factors to be identified explaining 51% and 20.9% of total variance, respectively (Table 10). The first factor (PC1) was highly and positively associated with the largest insoluble polymers (F1*), the medium size soluble polymers (F2), the largest glutenin polymers (both insoluble and soluble; F1*+F1) and with the polymeric fraction (F1*+F1+F2). Moreover, it was highly and negatively related with the small oligomers fraction (F3), the monomeric gliadin fraction (F4), the total monomeric fraction (F3+F4) and mon/pol ratio. Thus, PC1 could be considered a factor linked to the

degree of polymerization, mostly depending on the capacity to form covalent bonds. The second factor (PC2) was positively associated with gluten index (depending on the interactions among gluten proteins, both gliadins and glutenins), with the largest insoluble polymers (F1*) and with %UPP (depending on glutenin polymers size and amount) and negatively related with grain protein content (that can affect mostly gliadin accumulation) and the large size soluble polymers (F1) (that affect negatively %UPP). Thus, PC2 could be considered as a "gluten proteins assembly" factor, including the different interactions occurring in the gluten network. Both the factors linked to the degree of polymerization and the gluten proteins aggregation are major determinants of technological quality.

Table 10. Loading matrix values for the first two principal components (PC1 and PC2), considering the original variables. The corresponding percentages of accounted variation are also reported.

Original Variables	Loading Matrix Values	
	PC1	**PC2**
Grain protein content	0.09	−0.57
Gluten index	−0.21	0.47
F1* (%)	0.72	0.64
F1 (%)	0.29	−0.80
F2 (%)	0.54	−0.35
F3 (%)	−0.57	−0.26
F4 (%)	−0.81	0.29
F1*+F1 (%)	0.94	0.04
F1*+F1+F2 (%)	0.99	−0.09
F3+F4 (%)	−0.99	0.09
UPP (%)	0.55	0.77
mon/pol	−0.99	0.10
Percentage explained variation	**51**	**20.9**
Percentage cumulative variation	**71.9**	

In Figure 1, the biplot relative to the principal component analysis is reported. Based on the barycenter of the categorical variables (Figure 1, yellow marks), the nitrogen level did not show a clear separation along the two factors considered. On the contrary, the separation between the two years was observed mainly along the "gluten proteins assembly" factor (PC2) with the 2018 in the lower part. However, the separation between the crop seasons has to be interpreted also considering the genotype behaviors. Only for Quadrato the two years were separated mainly along the PC1 (polymerization degree factor), with the 2018 showing the positive and higher values. No clear separation was observed for the early maturing genotype Redidenari along the two PC factors. On the other hand, Marco Aurelio and Pietrafitta showed a clear separation of the two years only along the PC2, more marked for Marco Aurelio, with the 2018 showing the lower values. Finally, only the two genotypes, Marco Aurelio and Pietrafitta were clearly separated along PC1, presenting Marco Aurelio positive values and RDD negative values.

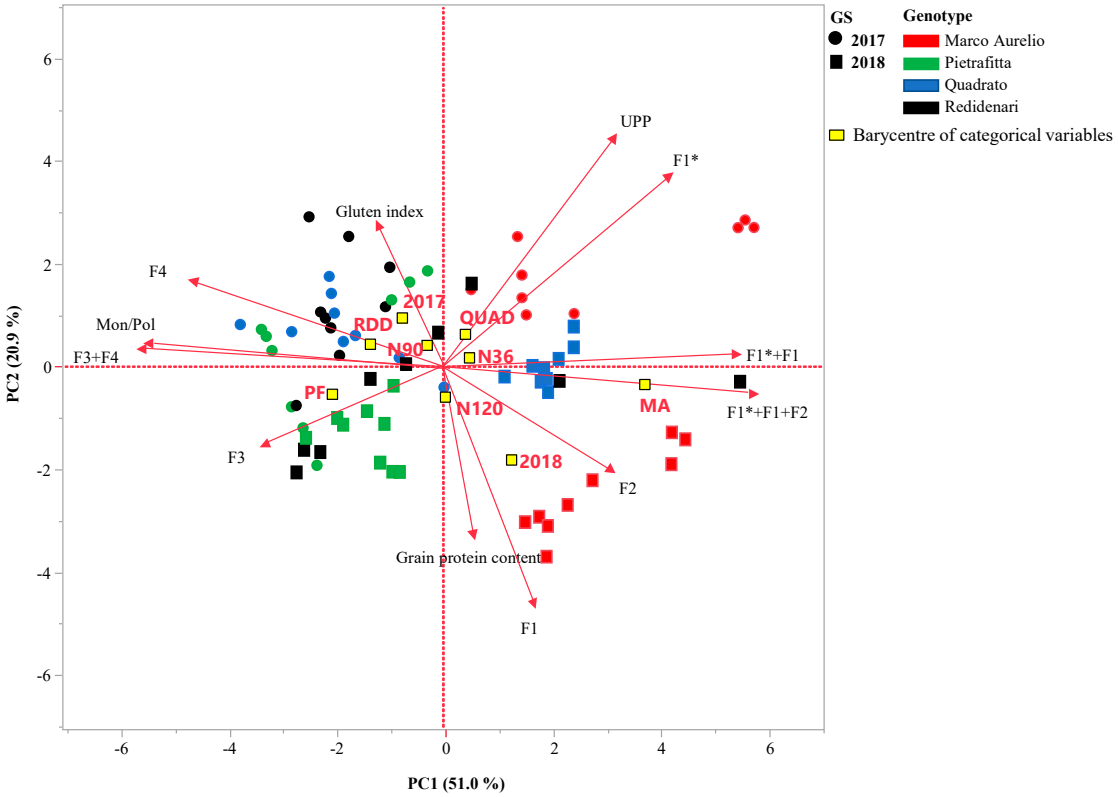

Figure 1. Biplot relative to the principal component analysis performed on grain protein content, gluten index, SDS insoluble (F1*) and soluble protein fraction (F1–F4) separated by SE-HPLC, monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein. In yellow, the barycenter of the categorical variables, growing season (2017 and 2018), genotype (MA, Marco Aurelio; PF, Pietrafitta; QUAD, Quadrato; RDD, Redidenari) and nitrogen level (N36, N90 and N120) are shown.

4. Discussion

In the Mediterranean climate, the rainfall variability together with the frequency of high temperature during the grain filling period, may cause large fluctuations in durum wheat grain yield and technological quality aspects [3,37]. In semi-arid regions, a further increase in temperatures together with reduced rainfall are expected following the ongoing climate change [38,39]. This trend will influence also the crop responses to nitrogen fertilization, which depend on rainfall amount and distribution during the crop cycle, to the amount and timing of nitrogen applications as well as to the initial soil nitrogen levels [40,41]. Moreover, Malik et al. [33] highlighted that the combinations of cultivars, nitrogen and temperature are needed to explain the variation in the quantity and size distribution of the polymer proteins and their effect on the quality of the end-product. To the best of our knowledge, for durum wheat, this type of information is still lacking. The results obtained in this study represent a tile of the complex mosaic depicting the interactions among environment, fertilization and genotype.

Glutenin polymers are among the major determinants of wheat quality. Polymers are formed by different types of subunits that are functionally divided into chain terminators, chain extenders, and chain branches, according to their possibility to form one, two, or three (or more) intermolecular bonds, respectively (reviewed in [23]). The combination of these three functional glutenin classes gives rise to a range of glutenin polymers with different sizes and structures, that contributes to dough rheological properties. In general, the higher the size and amount of glutenin polymers, the better dough strength, that can be predicted by the %UPP value [7].

In our experimental condition, the two growing seasons showed a different climatic trend in terms of rainfall distribution and temperatures. Significant lower yield and thousand kernel weight, together with higher protein concentration were observed for all the genotypes in 2018, characterized by higher temperatures during the grain filling with respect to the first growing season. Moderate high temperature during grain filling, between 25 °C and 35 °C, and short periods of very high temperature (>35 °C) at the end of grain filling phase, as those we observed in the second growing season, are frequently associated with a decrease in grain yield and an increase in grain protein concentration [8,42]. However, the genotypes Marco Aurelio and Redidenari (released in 2010 and 2015, respectively) were less influenced by the growing season with respect to Quadrato and Pietrafitta (both released in 1999). The positive effect of nitrogen fertilization was clearer for the protein content than for grain yield as also reported in literature under Mediterranean climate [43–46]. However, the high yield response observed for Redidenari under N90 level was particularly interesting, indicating the possibility of limiting nitrogen inputs by adopting genotypes capable to optimize the use of nitrogen.

The growing season differently affected the gluten index, an indicator of gluten strength for durum wheat, in relation to the genotypes, showing only Marco Aurelio and Redidenari lower values in the warmer year. In bread and soft wheat, dough strength has been often positively correlated with the proportion of UPP [15,18,47–49]. As for durum wheat, the relation between %UPP and gluten index has been less investigated. In our experimental condition, this relation was genotype dependent, since only Marco Aurelio and Redidenari showed simultaneous decrease of gluten index and %UPP in the second year.

The composition and functionality of storage proteins have been significantly affected by growing season and genotype, while the effect of N fertilization level was rather small (Table S2) [50] as also resulted by PCA analysis. Several studies reported an increase in the proportions of the monomeric gliadins with increasing N availability [26,27]. In our experimental conditions, this was true only for the genotype Redidenari due to an increase of F4 component represented mainly by α/β type gliadin. An interesting result was the increase of %UPP for both Marco Aurelio and Redidenari under N90 level due to the increase of the F1*. The significant decrease of the larger insoluble polymers fraction (F1*) and %UPP observed in the second growing season for Marco Aurelio and Pietrafitta has to be discussed in relation to their earliness. Indeed, the very high temperature recorded at the end of the crop cycle (3 days with T > 35 °C) could have negatively influenced these two genotypes that are medium and medium-late maturing genotypes. This result is probably due to the fact that the assembly of the storage proteins takes place at the end of the grain filling phase [10,51,52]. Shewry et al. [53] proposed that at the end of the cycle, the loss of water favors the polymer chains contact inducing the assembly through disulphide crosslinking or through inter-chain hydrogen bonding. The effect of the temperatures on gluten protein assembly, have been studied mostly in bread wheat and only few studies are available for durum wheat. In common wheat, several research studies suggested that moderate high temperature or few days of very high temperature resulted in a significant reduction in the proportion of the SDS-insoluble protein fraction [15,17,47]. Other studies showed that the size of the glutenin polymers increased in response to short periods of very high temperature [18]. Ferreira et al. [8], in durum wheat, reported also a positive effect of the high temperature during the whole grain filling period on gluten protein assembly. Thus, the relationship between the gluten protein assembly and high temperatures is still not clear and needs more investigation. In our experimental conditions, in the second growing season, the two late maturing genotypes (Marco Aurelio and Pietrafitta), together with the decrease in F1* and %UPP showed an increase of both F2 and F1 fraction, the latter together with the other genotypes, confirming that the synthesis of the SDS soluble polymers continued also under high temperature condition [14,47]. Due to the concurrent decrease in F1* and increase in F1 and F2 fractions, Marco Aurelio and Pietrafitta did not significantly change their polymeric fraction between the two years. The increase of both %UPP and polymeric fraction observed in Quadrato and only of polymeric fraction observed in Redidenari in the second growing seasons is also linked to their earliness. Indeed, it seems like that on these genotypes, which are medium-early and early

maturing, respectively, only the moderately high temperatures occurring during the grain filling acted, but not the extreme ones recorded at the end of the crop cycle. Indeed, also the results of the PCA highlighted the negative effect of the extreme temperatures on the gluten proteins assembly properties (PC2) only for Marco Aurelio and Pietrafitta, while for Quadrato a separation of the values only along the polymerization degree factor (PC1) was observed, with the warmer year showing the positive and higher values.

Because %UPP depends on protein distributions among the four areas typically used for its calculation, with the chain branchers and extenders mostly present in the fractions F1 (in particular F1*) and F2, it is important not only to select durum wheat varieties with proper glutenin compositions able to give rise to polymers of adequate size and amounts, but also that are synthetized in periods less susceptible to environmental changes, such it has occurred here for the medium early and early maturing varieties.

5. Conclusions

In the two growing seasons, the four durum wheat genotypes showed different capacities of the gluten proteins to assembly in a visco-elastic structure in relation to their earliness. In particular, in the second warmer year the late maturing genotype, Marco Aurelio and Pietrafitta showed a significant decrease of larger insoluble polymers fraction (F1*) and %UPP with a negative effect on their protein assembly level, despite Marco Aurelio always showed higher degree of polymerization. On the contrary, the medium-early and early maturing genotypes Quadrato and Redidenari, probably due to their earliness, did not change their "protein assembly level" in relation to the growing season.

The effect of N fertilization on the gluten protein polymerization and assembly was rather small, but among the N levels utilized the increase of F1*, %UPP and monomeric fraction under N90 was observed. Moreover, also the highest yield and gluten index values were obtained under N90. This was true especially for Redidenari.

In general, the effect of the growing season on the parameters evaluated was more evident than those of genotype and nitrogen level.

The results obtained in this study regarding four durum wheat genotypes clearly indicate different patterns of protein assembly in relation to the growing season, a factor that has a great influence on quality characteristics, thus contributing to the rational selection of the durum wheat genotypes, in particular those to include in supply chains, suitable for facing the ongoing climate changes in Mediterranean area.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/5/755/s1: Table S1. Mean square of effects (year, Y; genotype, G; nitrogen level, N) resulting from analysis of variance (ANOVA) performed on yield and technological parameters. Table S2: Mean square of effects (year, Y; genotype, G; nitrogen level, N) resulting from analysis of variance (ANOVA) performed on sonicated protein fraction (F1*) and SDS-soluble protein fraction (F1–F4) separated by SE-HPLC, monomeric/polymeric ratio (mon/pol) and proportion of unextractable polymeric protein (UPP). Figure S1. Rainfall distribution and maximum and minimum mean temperatures for the two growing seasons 2017 (a) and 2018 (b). Figure S2. SE-HPLC chromatograms of SDS-extractable protein fraction (a) and of SDS-unextractable protein fraction (b).

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