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Gluten Protein Compositional Changes in Response to Nitrogen Application Rate

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Abstract: Field trials were conducted to evaluate the effect of nitrogen level on wheat protein content and composition in 16 cultivars over two years at three locations. The nitrogen treatment comprised two nitrogen levels, 0 kg ha^{-1} as low and 100 kg ha^{-1} as high nitrogen, applied as top dressings of 50 kg nitrogen per ha at tillering and stem extension growth stages. Increased nitrogen level generally enhanced grain protein by 11.3% (11.5% vs. 12.8%). Considering protein composition determined by reversed phase–high-pressure liquid chromatography, higher nitrogen supply generally enhanced the proportion of total gliadins, α-gliadins, γ-gliadins and high-molecular-weight glutenin subunits by 1.1%, 2.0%, 3.7%, 0.6% and 0.9%, respectively, and reduced albumins and globulins, ω-gliadins, total glutenins and low-molecular-weight glutenin subunits by 1.1%, 1.7%, 1.9% and 3.2%. Under a high nitrogen level, the historical cultivars Libellula, San Pastore and U-1 had a higher protein content (13.1–15.2%) with significantly higher total gliadins, which resulted in a significantly higher gliadin/glutenin ratio (1.68–1.92). In the modern cultivars, protein content varied between 11.4% and 14.6% with a well-balanced gliadin/glutenin ratio (1.08–1.50), except for cultivar MV Nemere which had a high gliadin/glutenin ratio at both nitrogen levels (1.81 vs. 1.87). In summary, increasing nitrogen level enhanced grain protein content while the composition of gliadin and glutenin fractions was changed to a lesser extent and was largely cultivar specific and therefore should be considered for wheat baking quality assessment and breeding purposes.

Keywords: wheat; cultivars; nitrogen fertilization; environment; proteins; reversed phase–high-pressure liquid chromatography

1. Introduction

Among dominant grain cereals produced for human food consumption, common wheat (*Triticum aestivum* L.) is one of the most important. Yield and quality of wheat grain are dependent on genotype, soil fertility, growing conditions and crop nutrition. Bread wheat yield potential is largely genetically determined and varies significantly as a result of differences in climate and adequate production systems, which include mineral fertilization [1,2]. Utilization of nitrogen fertilization has many benefits. Nitrogen (N) is necessary for wheat growth, and crop yield and quality depend upon a sufficient input of N. A satisfactory supply of N is essential for the grain protein accumulation that is necessary for baking and processing quality [3,4]. Grain protein content is an important factor defining wheat end-use quality and usually varies between 6 and 19% and has an impact on the grading of flour quality for traditional bread making and other food products [5,6]. In Croatia, in accordance with the "Regulation on quality parameters and quality classes of wheat in the purchase of wheat (2018)", in the formation of the market

price, the final and decisive parameter is protein content (%) and, accordingly, wheat produced exclusively for purchase in the territory of the Republic of Croatia is classified into four qualitative classes: premium (>15%), I class (13.5–14.99%), II class (12–13.49%), III class (10.5–11.99) and IV class (<10.49%). According to their solubility characteristics, wheat grain proteins are classified as albumins and globulins (AGs), gliadins (GLIs) and glutenins (GLUs). GLIs and GLUs are the two main groups of gluten proteins, the endosperm storage proteins, accounting for 60~80% of the total grain proteins. Gluten proteins influence dough rheological properties that determine the processing quality of bread, noodles and cookies [7–9]. GLIs belong to the monomeric prolamin fraction, and are classified into three structural groups of polypeptides according to their electrophoretic mobility, α/β-, γ- and ω-GLI. The polymeric glutenin fraction is constituted of high-molecular-weight (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) linked with intermolecular disulphide bonds. The gluten protein accumulation during the grain filling period is largely influenced by environmental growing conditions, particularly N availability. However, the type and distribution of gluten subunits have a strong genetic component [10,11]. It is reported that the composition of the protein fractions is more influenced by the N nutrition than by temperature [12].

Grain nitrogen content plays an important role affecting the ratio of storage to non-storage proteins, the percentage of the storage protein fractions and, thus, the GLI/GLU ratio and the ratio of HMW-GS/LMW-GS [8,11,13]. To obtain the maximum wheat potential in the field, N is the most limiting nutrient required by plants. The growth of wheat plants begins with protein reserves of the kernel, which serve to maintain the germination and further growth of the seedling until the first leaf appears. After rootlets are developed, additional N is obtained by the rootlet system and at this stage the N uptake is limited by root size. In the Northern Hemisphere, it is considered necessary to administer a first application of N fertilizer before the second leaf emerges. A second important time for N application is the tillering stage, when the N concentration in the tissue impacts the formation of the tillers in each plant. The third N application is the most important and it is critical for biomass formation during the extension stage and later for promoting protein accumulation [4,13].

In Croatia, the annual wheat production was 820,682 t in the last 10 years (2009–2018), with 5.12 t ha^{-1} [14]. Since producers in Croatia get a higher price for premium quality wheat (grain protein content above 15%), it is desirable to select high-quality cultivars that offer a competitive advantage by minimizing yield penalty in environments that are not classified as high yielding. A few studies regarding bread-making quality and environmental influence on gluten proteins are being done continuously. However, comprehensive research on nitrogen's influence in different environments on protein content and composition has not been carried out so extensively. Therefore, the hypothesis and objective of this study is to determine the quantitative changes of quality-related proteins in historical and currently grown wheat cultivars grown in different agro-ecological conditions at two N fertilization levels in order to assess if there is a specific cultivar reaction with nitrogen treatment in terms of their quality potential for breeding and technological purposes.

2. Materials and Methods

2.1. Plant Materials and Field Experiments

Sixteen winter wheat cultivars were evaluated in field trials under two N fertilization levels during two consecutive seasons (2016/17 and 2017/18) at three locations (Osijek, Zagreb and Poreč) representing different agro-ecological conditions and soil types in Croatia. The panel was assembled to represent the historical and current significance of cultivars in production in Croatia and their parental contributions. The cultivar list, year of registration and their country of origin and breeding institution are listed in Table 1.

Each of the six field experiments was set up in a split-plot factorial design in three replications with N fertilization levels as the main plots and 16 wheat cultivars as sub-plots. The harvested plot size was 7.56 m^2 at Osijek and Poreč and 4.95 m^2 at Zagreb. Seeding

level was 350 kernels m^{-2} in all trials and for all cultivars. Buffer plots were planted between the main N treatment plots. Basic fertilization before sowing of 74 kg N ha^{-1}, 80 kg P$_2$O$_5$ ha^{-1} and 120 kg K$_2$O ha^{-1} was applied by adding 100 kg ha^{-1} of urea (46% N) and 400 kg ha^{-1} NPK (7:20:30).

Table 1. List of wheat cultivars, year of registration, country of origin and breeding institution.

Cultivar	Year of Registration	Country */Breeding Institution
U-1	1936	HR/Agricultural Institute Osijek
SAN PASTORE	1940	IT/Istituto nazionale di genetica per la cerealicoltura
BEZOSTAYA-1	1959	RU/KRIA P.P. Lukyanenko
LIBELLULA	1965	IT/Istituto Nazionale di Genetica per la cerealicoltura
SRPANJKA	1989	HR/Agricultural Institute Osijek
ISENGRAIN	1997	FR/Florimond Desprez Veuve et Fils
BOLOGNA	2001	FR/ETS CC BENOIST
GRAINDOR	2006	FR/Unisigma GIE (FR)
INGENIO	2008	EU/Syngenta AG Participation
OS OLIMPIJA	2009	HR/Agricultural Institute Osijek
SOLEHIO	2009	FR/SARL Adrien Momont et Fils
KRALJICA	2010	HR/Agricultural Institute Osijek
VIKTORIA	2011	HR/Agrigenetics d.o.o.
MV NEMERE	2013	HU/MTA Agrartudomanyi Kutatokoezpont
SOFRU	2013	FR/Caussade Semences S.A.
BC LJEPOTICA	2016	HR/BC Institute Zagreb

* HR—Croatia, HU—Hungary, FR—France, RU—Russia, IT—Italy.

The N treatment comprised two N fertilization levels, 0 kg N ha^{-1} as low N and 100 kg N ha^{-1} as high N, applied as top dressings of 50 kg N ha^{-1} at tillering (GS23-25 after Zadoks et al. (1974)) and stem extension (GS33-35) growth stages. All other cultural practices, including application of herbicides, insecticides and fungicides to control major weeds, insects and foliar diseases, were typical for commercial wheat production in Croatia. Total N fertilizer application in commercial wheat production varies from 120–180 kg of N ha^{-1}. Descriptions of the soil type, soil N content and N fertilization levels for N0 and N100 treatments for the six location–season combinations are given in Table S1 (Supplementary Materials).

Osijek and Zagreb locations are classified as having a moderately warm and rainy oceanic climate (Cfb), while the Poreč location is classified as having humid subtropical climate (Cfa) by the Köppen and Geiger climate classification [15]. Basic meteorological data for all locations for 2016/17 and 2017/18 and multi-annual averages are provided in Supplementary File Table S5. On average, during both seasons, Osijek was the driest and coolest site, while Poreč was the warmest and had the highest amount of rainfall. Zagreb had the highest amount of precipitation and the lowest average temperature only in the 2017/18 season (Table S5, summary in Supplementary Materials).

2.2. Protein Analysis

2.2.1. Grain Protein Content

Replicate grain samples of 0.5 kg were collected after the harvest and were polled and homogenized and used further for protein analysis. Grain protein content (P) (N × 5.7, DM) was measured using an Infratec 1241 Grain Analyzer (Infratec 1241, Foss Tecator, Hillerød, Denmark).

2.2.2. Wet Gluten Content and Gluten Index

Wet gluten content (WG) and gluten index (GI) of approximately 70% extraction flour (Quadrumat Senior mill, Duisburg, Brabender, Germany) were analyzed using a Glutomatic 2200 (Perten Instruments, Hagersten, Sweden) according to ICC standard No. 155.

2.2.3. Extraction of Proteins

Prior to the reversed phase–high-pressure liquid chromatography (RP-HPLC) analysis, the single protein fractions were sequentially extracted according to Wieser et al. [12] with some modifications. Briefly, 50 mg of wholemeal flour (Retsch Centrifugal Mill ZM1, Haan, Germany, equipped with 1 mm sieve) were extracted by 1 mL of 0.4 M NaCl for 30 min at RT (albumin/globulin (AG) extract). From the remaining pellet, the gliadins (GLIs) were extracted with 1 mL of 50% 1-PrOH for 60 min at RT and, finally, the glutenins (GLUs) were extracted with 1 mL of a solution containing 50% (*v/v*) 1-PrOH, 2 M urea, 1% (*w/v*) dithioerythritol and 0.05 M Tris-HCl (pH 7.5) for 60 min at 60 °C. During all extractions, the suspensions were vortexed for 1 min every 10 min and then centrifuged for 15 min at 14,000 rpm and RT. The obtained supernatants were stored at −20 °C and filtered through a 0.45 μm PVDF syringe filter prior to HPLC analysis. Two extractions were performed for each sample.

2.2.4. HPLC Analysis of Proteins

Extracted wheat proteins were analysed according to the method of Wieser et al. [12] using high-performance liquid chromatography (HPLC) (Perkin Elmer Instruments, Waltham, MA, USA) coupled with Total-Chrom software and a photodiode array detector. Elution was performed with linear gradient of acetonitrile (ACN/0.1%TFA) in water (H$_2$O/0.1%TFA) from 24–54% in 30 min at flow rate of 1 mL min^{-1} and column temperature of 50 °C. Proteins were separated on a C18 reverse phase column (5 μm 4.6 × 150 mm; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Quantification of protein fractions was based on measuring their peak area at 210 nm. The peak areas under AG, GLI and GLU chromatograms were used for further calculations of their relative proportion (%) in total extracted proteins.

2.3. Statistical Analysis

All statistical analysis was done in R (R Core Team 2013). Two linear models were employed as described by Cormier et al. [16]. Firstly, in all field trials (where N levels, year and location of testing were separated), least square means were calculated using cultivars as fixed factors. These adjusted means were used in the subsequent analyses using the following linear model:

$$Y_{ijl} = \mu + N_l + GEN_i + LOC_j + YEAR_k + N_l : GEN_i : LOC_j : YEAR_{ki} + \varepsilon_{ijkl}$$

Y_{ijl} is the phenotype of genotype i in location j and year $_k$ at N treatment level l (0 kg/ha and 100 kg/ha of N applied as top dressing), μ is the general mean, N_l is the effect of N, GEN_i is cultivar (genotype) effect, LOC_j is the effect of environment (3 levels), $YEAR_k$ is the year effect (2 levels), $N_l : GEN_i : LOC_j : YEAR_k + \varepsilon_{ijkl}$ is the interaction term for all possible combinations and ε_{ijkl} is residual error terms.

The differences among cultivar means were tested using Tukey's HSD test (library *emmeans)*. The statistical significance of the differences among main factors and treatments was tested using an LSD test at the level of probability $p < 0.05$.

3. Results and Discussion

The results of ANOVA analysis are shown in Table S2 (Supplementary Materials). For all analysed traits, statistically significant differences were found for the main factors (cultivars, N level, year and location) and their interactions, which is in accordance with the results of other authors [17–19]. Among the main sources of variation, cultivar was the main contributing factor for the majority of quality-related proteins, therefore, in the data presentation, we further emphasized the differences between cultivars grown under different N treatments. Grain and flour quality of wheat cultivars under low and high N rates are presented in Table 2. The mean values of the P and WG content for 16 wheat cultivars under low N were relatively low, with means of 11.5% and 22.5%, respectively.

Under same low N application, the examined cultivars expressed relatively high gluten strength, with an average GI value of 95. The cultivars OS Olimpija and U-1 showed the highest grain P (13.4% and 13.0%, respectively), while cultivars Graindor, Solehio and Sofru had the lowest (9.9%, 10.2% and 10.3%, respectively). WG ranged from 17.2% (Isengrain) to 26.6% (OS Olimpija), while GI ranged from 89 (U-1) and 99 (Graindor and BC Ljepotica). The P and WG contents were significantly higher under high N levels and were cultivar dependent (Table 2 and Table S2). Generally, the P (11.5% vs. 12.8%) and WG (22.2% vs. 25.8%) increased by 11.3% and 16.2%, while GI (95 vs. 92) decreased by 3.2%, which is in agreement with Zheng et al. [20], who also found that processing quality parameters, except gluten index, were remarkably improved after increasing N application. These authors noted that protein content of the four cultivars and four N levels (N_0–N_{75}–N_{150}–N_{225}) increased during two consecutive growing seasons from 10.2% to 13.9% and 9.5% to 12.2%, respectively. Yu et al. [21], studying six Australian bread wheat cultivars grown under different N treatments over two years at several locations, noted that the genotype was dominant in grain P content in response to N availability, indicating the diversity of cultivars used in the experiment.

Table 2. Mean [1] values of grain and flour quality in wheat under low and high N levels.

CULTIVARS	P_{DM} [2] (%)		WG (%)		GI	
	N_0 [3]	N_{100}	N_0	N_{100}	N_0	N_{100}
U-1	13.0 ± 1.6 [b4]	15.2 ± 1.1 [a]	26.3 ± 3.7 [ab]	29.2 ± 4.8 [b]	89 ± 7.5 [e]	84 ± 18.1 [i]
SAN PASTORE	11.6 ± 1.4 [f]	13.1 ± 1.2 [f]	22.8 ± 4.5 [e]	26.6 ± 3.5 [e]	91 ± 8.2 [d]	86 ± 16.6 [hi]
BEZOSTAJA-1	12.5 ± 1.6 [c]	13.7 ± 1.0 [d]	26.0 ± 2.5 [b]	30.3 ± 4.1 [a]	94 ± 2.4 [c]	81 ± 14.8 [j]
LIBELLULA	11.8 ± 1.7 [e]	13.4 ± 0.8 [e]	25.7 ± 6.0 [c]	28.5 ± 4.1 [c]	90 ± 8.3 [d]	82 ± 12.9 [j]
SRPANJKA	11.7 ± 1.6 [ef]	13.0 ± 0.7 [g]	21.9 ± 5.2 [f]	25.6 ± 4.3 [f]	98 ± 2.2 [ab]	95 ± 9.6 [de]
ISENGRAIN	10.8 ± 1.5 [h]	11.5 ± 1.0 [k]	17.2 ± 4.5 [j]	20.5 ± 2.6 [k]	94 ± 10.4 [c]	99 ± 0.0 [a]
BOLOGNA	11.6 ± 1.6 [ef]	12.5 ± 1.1 [h]	22.7 ± 5.3 [e]	27.3 ± 1.9 [d]	93 ± 12.6 [c]	94 ± 13.0 [ef]
GRAINDOR	9.9 ± 1.7 [k]	11.8 ± 0.8 [j]	18.0 ± 5.1 [i]	22.7 ± 3.2 [h]	99 ± 0.8 [a]	98 ± 1.3 [a]
INGENIO	10.9 ± 1.5 [h]	12.1 ± 1.0 [i]	21.5 ± 5.1 [fg]	24.8 ± 1.5 [g]	98 ± 2.1 [ab]	97 ± 4.1 [ab]
OS OLIMPIJA	13.4 ± 1.9 [a]	14.6 ± 1.2 [b]	26.6 ± 5.3 [a]	30.5 ± 4.2 [a]	95 ± 6.5 [c]	88 ± 9.7 [g]
SOLEHIO	10.2 ± 1.4 [j]	11.4 ± 0.9 [k]	17.8 ± 4.4 [i]	22.4 ± 4.3 [hi]	98 ± 3.7 [b]	99 ± 1.4 [a]
KRALJICA	12.2 ± 1.4 [d]	13.7 ± 0.9 [d]	23.1 ± 5.5 [e]	27.4 ± 2.0 [d]	95 ± 3.6 [c]	92 ± 8.3 [f]
VIKTORIJA	12.6 ± 1.8 [c]	14.0 ± 0.8 [c]	24.5 ± 6.7 [d]	28.1 ± 2.9 [c]	94 ± 8.9 [c]	96 ± 2.6 [cd]
MV NEMERE	11.2 ± 1.5 [g]	12.1 ± 0.4 [i]	21.2 ± 4.1 [g]	25.2 ± 2.7 [fg]	95 ± 7.3 [c]	87 ± 8.8 [gh]
SOFRU	10.3 ± 1.3 [i]	11.4 ± 0.7 [k]	21.2 ± 4.4 [g]	21.6 ± 5.1 [j]	98 ± 1.7 [ab]	95 ± 7.5 [de]
BC LJEPOTICA	10.8 ± 1.1 [h]	11.9 ± 1.0 [j]	18.5 ± 4.9 [h]	22.0 ± 2.8 [ij]	99 ± 0.8 [a]	97 ± 2.4 [ab]
MEAN	11.5 ± 1.8	12.8 ± 1.5	22.2 ± 5.5	25.8 ± 4.5	95 ± 6.8	92 ± 11.0
LSD$_{GEN}$ 0.05	0.14	0.13	4.35	4.07	7.62	10.92

[1] Means ± standard deviation across years and locations under low and high N levels. [2] P = grain protein content (%), WG = wet gluten (%), GI = gluten index. [3] N_0 = low N level, N_{100} = high N level. [4] For each cultivar within a column, values followed by different letters are significantly different at $p < 0.05$.

The modern cultivars (bred after 1960 in a phase of the Green Revolution) (Table 1), OS Olimpija, Viktorija and Kraljica, with the highest P, WG and GI, have shown the greatest bread-making quality potential and their increase in the P content under a high N level was 8.8%, 12.5% and 11.0% (Table 2) and, in accordance with Croatian regulations, belong to the quality class I. It is important to note that all cultivars, except Isengrain and BC Ljepotica, achieved a higher quality class due to the increase in N intake. It is interesting to note that old cultivars (cross-bred until the late 1960s), Libellula, San Pastore and U-1, with P content of 11.6–13.0%, showed a significantly ($p < 0.05$) lower GI (89–91) than the modern ones, with a GI from 93 to 99. These cultivars also showed a remarkable increase in P content with increasing N level by 14%, 13.2% and 17.1%, respectively. The old cultivar Bezostaja-1 with 13.7% P under higher N showed the largest decrease in GI (94 vs. 86) as well as cultivars MV Nemere (95 vs. 87) and Libellula (90 vs. 82), meaning that there was a significant decrease in gluten strength under higher N level, which could negatively

affect the baking properties of flour. Guerrini et al. [22] noted that flour from the old wheat cultivars is typically characterized by high yield and poor technological properties and is not suitable for industrial baking but, recently [23], the old wheat cultivars have been reintroduced in wheat breeding programs as a contribution to biodiversity and nutritional and nutraceutical properties.

As we mentioned before, the protein accumulation during the grain filling period is largely influenced by N fertilization level, however, the composition of protein fractions has strong genetic background [19,24–26]. AG represents approximately 15–20% of the grain proteins and often has structural and metabolic functions with limited impact on the baking properties [27]. The AG proportion under low N ranged from 13.8% (OS Olimpija) to 22.0% (Isengrain). In relation to N increase (Figure 1), the AG mean generally decreased by 1.1%, but the N effect on cultivars was not consistent, so, in Bologna, Isengrain and San Pastore, the AG proportion decreased by 15.3%, 11.4% and 10.9%, while in Bezostaje-1, Ingenio and Solehio, it increased by 7.2%, 8.4% and 7.5%, respectively.
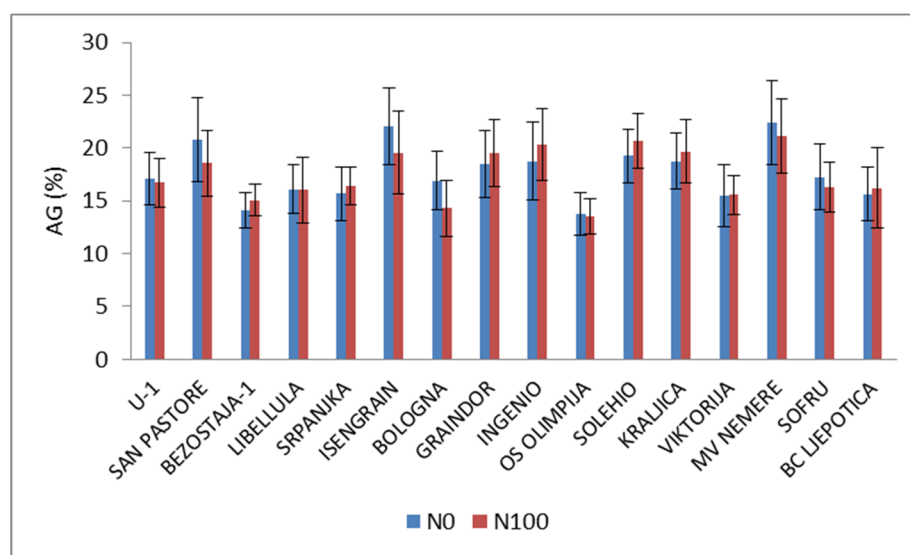

Figure 1. Proportions of albumins and globulins (AG) in total wheat grain protein under low (N0) and high (N100) N levels. (Histograms are showing mean values ± standard deviation across years and locations).

Some authors [12,17,28] noted that AG remained nearly unaffected under the higher N level, while Rekowski et al. [29] reported that late N supply significantly increased AG by 8–9%.

Storage GLI and GLU fractions constitute 80–85% of total grain proteins and contribute to rheological, pasting and textural properties of dough [27]. Polymeric glutenins predominantly define the dough elasticity while monomeric gliadins are responsible for dough extensibility [30–32]. Significant variability among cultivars under a low N level was observed in the proportion of GLI fractions, with the cultivar Isengrain having the lowest proportion of total GLI (40.0%) and the cultivar U-1 having the highest (55%). The proportion of minor ω-GLI ranged from 4.0% (Sofru) to 9.4% (Kraljica), while the proportion of major α-GLI ranged from 20.4% (Isengrain) to 29.5% (U-1) (Table 3). All GLI fractions were affected by the higher N supply and, generally, the total GLI (46.1% vs. 47.0%), α-GLI (24.2% vs. 25.1%) and γ-GLI (16.0 vs. 16.1) increased by 1.9%, 3.7% and 1.3%, respectively, while ω-GLI (5.9 vs. 5.8) decreased by 1.7%, meaning that the dominant α-GLI reacted the most to the N level increase (Table 3). Hurkman et al. [33] and Rossmann et al. [26] found that α-GLI and ω-GLI proportions increased under higher N levels, while the γ-GLI proportion decreased. Variations in composition of GLI fractions were strongly cultivar dependent (Table 3 and Table S3 in Supplementary Materials), with

the cultivars San Pastore and Sofru achieving the largest increase in total GLI (6.3% and 5.5%, respectively), while in cultivar U-1, the total GLI proportion decreased by 1% under a higher N level. A similar inconsistency was observed for other GLI factions.

Table 3. Mean [1] values of GLI proportions in total grain protein of wheat under low and high N levels.

CULTIVARS	GLI [2] (%)							
	Total GLI		ω-GLI		α-GLI		γ-GLI	
	N_0 [3]	N_{100}	N_0	N_{100}	N_0	N_{100}	N_0 [3]	N_{100}
U-1	55.0 ± 2.6 [a4]	54.5 ± 2.4 [a]	6.1 ± 2.0 [ah]	4.0 ± 0.8 [ai]	29.5 ± 1.6 [a]	30.5 ± 2.0 [a]	20.5 ± 1.9 [a]	20.0 ± 1.1 [a]
SAN PASTORE	50.0 ± 1.8 [bc]	53.1 ± 4.7 [b]	6.5 ± 0.7 [c]	7.7 ± 2.3 [b]	27.0 ± 0.9 [b]	28.2 ± 2.6 [b]	16.5 ± 0.9 [c]	17.3 ± 1.1 [cd]
BEZOSTAJA-1	47.2 ± 4.6 [e]	47.3 ± 4.0 [e]	5.4 ± 2.3 [ef]	5.2 ± 1.0 [g]	25.6 ± 2.2 [c]	26.4 ± 2.6 [de]	15.6 ± 1.1 [def]	15.7 ± 1.2 [e]
LIBELLULA	51.1 ± 4.8 [b]	52.2 ± 5.9 [b]	5.9 ± 0.8 [g]	6.3 ± 2.4 [cd]	27.2 ± 3.2 [b]	28.4 ± 4.1 [b]	18.5 ± 1.9 [b]	17.6 ± 1.2 [c]
SRPANJKA	47.0 ± 4.2 [e]	48.3 ± 1.7 [de]	5.4 ± 0.8 [de]	6.0 ± 0.3 [e]	26.6 ± 2.8 [c]	27.1 ± 1.3 [cd]	15.4 ± 1.3 [ef]	15.2 ± 0.8 [efg]
ISENGRAIN	40.0 ± 2.4 [h]	41.8 ± 6.5 [j]	6.0 ± 1.8 [de]	6.4 ± 3.1 [c]	20.4 ± 2.3 [h]	21.1 ± 2.9 [h]	13.7 ± 1.1 [i]	14.3 ± 1.3 [h]
BOLOGNA	43.1 ± 2.7 [g]	44.5 ± 3.8 [g]	5.3 ± 2.3 [gh]	6.0 ± 1.3 [de]	22.4 ± 1.9 [efg]	23.0 ± 3.0 [g]	15.3 ± 0.8 [efg]	15.5 ± 2.2 [ef]
GRAINDOR	44.1 ± 3.9 [fg]	44.0 ± 4.2 [gh]	6.6 ± 3.0 [c]	5.2 ± 0.5 [g]	23.2 ± 1.8 [def]	23.7 ± 3.5 [g]	14.44 ± 0.6 [hi]	15.1 ± 2.4 [efgh]
INGENIO	42.6 ± 5.3 [g]	42.6 ± 2.0 [ij]	5.0 ± 0.4 [i]	4.4 ± 0.7 [h]	21.9 ± 4.3 [g]	21.4 ± 2.0 [h]	16.3 ± 1.2 [cd]	16.8 ± 1.0 [cd]
OS OLIMPIJA	48.9 ± 1.8 [cd]	48.7 ± 4.9 [d]	5.5 ± 0.4 [fg]	5.5 ± 1.0 [f]	27.5 ± 1.4 [b]	27.9 ± 3.4 [bc]	15.9 ± 1.5 [cde]	15.2 ± 1.1 [efgh]
SOLEHIO	42.8 ± 1.9 [g]	42.6 ± 4.3 [ij]	5.8 ± 0.8 [cd]	6.2 ± 1.3 [cde]	21.5 ± 1.2 [gh]	21.5 ± 2.4 [h]	15.2 ± 1.0 [fg]	14.9 ± 1.5 [efgh]
KRALJICA	47.9 ± 3.1 [de]	48.0 ± 3.1 [de]	9.4 ± 2.1 [a]	8.7 ± 2.2 [a]	24.0 ± 0.8 [d]	24.7 ± 1.1 [f]	14.6 ± 1.3 [gh]	14.6 ± 1.0 [gh]
VIKTORIJA	44.6 ± 2.5 [f]	45.8 ± 1.8 [f]	6.3 ± 0.7 [gh]	5.1 ± 0.6 [g]	24.3 ± 1.8 [d]	26.1 ± 1.5 [e]	15.8 ± 1.6 [cdef]	14.7 ± 1.0 [fgh]
MV NEMERE	49.7 ± 2.8 [bc]	51.1 ± 2.6 [c]	7.5 ± 1.1 [b]	7.5 ± 1.3 [b]	23.6 ± 1.5 [de]	24.7 ± 1.9 [f]	18.6 ± 1.5 [b]	18.9 ± 0.7 [b]
SOFRU	40.7 ± 3.5 [h]	43.0 ± 4.2 [hi]	4.0 ± 0.6 [i]	4.9 ± 1.3 [g]	22.2 ± 2.3 [fg]	23.6 ± 2.2 [g]	14.6 ± 1.5 [gh]	14.5 ± 1.7 [gh]
BC LJEPOTICA	42.8 ± 5.4 [g]	44.3 ± 5.5 [g]	4.4 ± 3.1 [e]	4.3 ± 0.5 [hi]	21.3 ± 2.7 [gh]	23.3 ± 4.2 [g]	15.6 ± 1.4 [def]	16.6 ± 1.6 [d]
MEAN	46.1 ± 5.3	47.0 ± 5.6	5.9 ± 2.0	5.8 ± 1.9	24.2 ± 3.3	25.1 ± 3.8	16.0 ± 2.2	16.1 ± 2.1
LSD$_{GEN0.05}$	1.5	1.1	0.4	0.3	1.2	1.0	0.7	0.9

[1.] Means ± standard deviation across years and locations under low and high N levels. [2] GLI = gliadins. [3] N_0 = low N level, N_{100} = high N level. [4] For each cultivar within a column, values followed by different letters are significantly different at $p < 0.05$.

The old cultivars Libellula, San Pastore and U-1 under both N levels achieved a significantly higher total GLI and α-GLI compared to the modern ones (Table 3). De Santis et al. [34], who studied only gluten proteins, without AG, reported that the old and modern Italian cultivars showed similar α/γ-GLI expression (61% vs. 58%), while a marked decrease in ω-GLI expression was observed in the modern ones (11% vs. 4%), which is not so clear among our cultivars (Table 3). The composition and quantity of the GLU are the major determinants of wheat bread-making quality because a higher content of GLU is related to an increase in gluten strength that allows the dough to maintain the desired final shape and maintains the desired appearance of the final baking products [31]. The total GLU proportion under a low level ranged from 27.7% (MV Nemere) to 42.0% (Sofru). The proportion of LMW-GS as a major GLU fraction ranged from 18.4% (San Pastore) to 30.8% (Sofru) and for minor HMW-GS from 7.8% (U-1) to 13.2% (Bologna) (Table 4).

Analysis of the data revealed significant effects of N levels on the GLU components and they were greatly cultivar dependent (Table 4 and Table S4 in Supplementary Materials). In relation to the N increase (Table 4), the HMW-GS mean increased by 0.9% (10.9% vs. 11.0%), while total GLU (36.2% vs. 35.5%) and LMW-GS (25.3% vs. 24.5%) decreased by 1.8% and 3.0%, respectively, meaning that major LMW-GS reacted most sensitively to increased nitrogen intake. These results are in agreement with others [26,33,35], who showed that proportions of low-S proteins (such as ω-GLI and HMW-GS) increase, while proportions of S-rich proteins (such as LMW-GS) decrease with increasing N fertilization rates. The largest decrease in the total GLU proportion under higher N was observed for the cultivars BC Ljepotica and Srpanjka (5.0% and 5.4%, respectively), while Bologna and U-1 showed an increase in GLU by 3.0%. It has been reported that HMW-GS have a crucial impact on wheat baking quality even though they constitute only 10% of the total grain P. Among cultivars, significant variations between low and high N levels were observed for HMW-GS, ranging from −6.6% (BC Ljepotica) to + 7.5% (U-1) (Table 4), which is consistent with others who found that the expression of storage proteins as well as HMW-GS is strongly associated with genotype [19,36]. Hurkman et al. [33] analyzed the effect of

different temperature regimes with and without fertilizer on individual gluten proteins (quantitative 2-DE) of one cultivar and concluded that under moderate temperature, the majority of individual protein spots of HMW-GS, ω-GLI and some α-GLI increased in the range of 25.2–49.2%, 56.0–107.6% and 21.5–50.2%, respectively, while two LMW-GS spots and a minor γ-GLI spot decreased by 23.1%, 31.4% and −28%, respectively. Xue et al. [37] reported that globulins, LMW-GS and α- and γ-GLI were more sensitive to split N application, while Zhong et al. [38] noted that α-GLI and HMW-GS were commonly dependent on nitrogen top dressing timing.

Table 4. Mean [1] values of GLU proportions in total grain protein of wheat under low and high N levels.

CULTIVARS	GLU [2] (%)							
	T GLU		HMW-GS		LMW-GS		GLI/GLU	
	N_0 [3]	N_{100}	N_0	N_{100}	N_{0c}	N_{100}	N_0 [3]	N_{100}
U-1	27.9 ± 1.5 [g4]	28.9 ± 3.2 [i]	7.8 ± 0.6 [h]	8.4 ± 1.2 [h]	20.0 ± 1.0 [f]	20.4 ± 2.0 [g]	1.98 ± 0.2 [a]	1.92 ± 0.3 [a]
SAN PASTORE	29.2 ± 2.8 [f]	28.3 ± 3.4 [ij]	10.8 ± 1.5 [ef]	10.6 ± 1.5 [f]	18.4 ± 1.9 [g]	17.7 ± 2.0 [h]	1.720.1 [c]	1.91 ± 0.3 [ab]
BEZOSTAJA-1	38.7 ± 4.3 [c]	37.7 ± 4.0 [de]	12.1 ± 3.1 [b]	11.4 ± 2.1 [bcd]	26.6 ± 3.1 [d]	26.2 ± 2.2 [e]	1.24 ± 0.3 [gh]	1.28 ± 0.2 [f]
LIBELLULA	32.9 ± 3.5 [e]	31.7 ± 3.9 [h]	10.7 ± 1.8 [ef]	10.8 ± 1.9 [ef]	22.1 ± 2.4 [e]	21.0 ± 2.1 [g]	1.58 ± 0.3 [d]	1.68 ± 0.3 [c]
SRPANJKA	37.3 ± 2.7 [d]	35.3 ± 2.6 [g]	10.6 ± 1.2 [f]	10.5 ± 1.2 [f]	26.8 ± 1.8 [d]	24.7 ± 1.5 [f]	1.27 ± 0.2 [fg]	1.38 ± 0.1 [e]
ISENGRAIN	38.0 ± 5.5 [cd]	38.7 ± 4.4 [c]	10.9 ± 1.8 [ef]	11.4 ± 1.8 [bcd]	27.1 ± 3.8 [d]	27.3 ± 2.9 [cd]	1.08 ± 0.2 [ij]	1.11 ± 0.3 [jk]
BOLOGNA	40.0 ± 4.6 [b]	41.2 ± 5.3 [a]	13.2 ± 2.3 [a]	13.8 ± 2.2 [a]	26.8 ± 2.5 [d]	27.4 ± 3.3 [c]	1.09 ± 0.2 [ij]	1.11 ± 0.2 [jk]
GRAINDOR	37.4 ± 4.0 [d]	36.5 ± 4.4 [f]	9.3 ± 2.0 [g]	9.3 ± 1.2 [g]	28.0 ± 2.3 [c]	27.2 ± 3.3 [cd]	1.20 ± 0.2 [h]	1.23 ± 0.2 [g]
INGENIO	38.6 ± 4.9 [c]	37.1 ± 2.1 [ef]	11.9 ± 3.1 [b]	11.8 ± 3.6 [bc]	26.7 ± 3.4 [bd]	25.3 ± 1.2 [f]	1.13 ± 0.3 [i]	1.15 ± 0.1 [hij]
OS OLIMPIJA	37.4 ± 2.3 [d]	37.9 ± 5.4 [d]	10.9 ± 1.1 [ef]	11.5 ± 2.0 [bcd]	26.5 ± 1.5 [d]	26.3 ± 3.5 [e]	1.31 ± 0.1 [f]	1.32 ± 0.3 [f]
SOLEHIO	37.9 ± 1.5 [cd]	36.7 ± 3.0 [f]	9.6 ± 1.1 [g]	9.2 ± 1.0 [g]	28.3 ± 0.7 [c]	27.5 ± 2.4 [c]	1.13 ± 0.1 [i]	1.17 ± 0.2 [hi]
KRALJICA	33.3 ± 2.4 [e]	32.3 ± 2.5 [h]	11.8 ± 1.4 [bc]	11.4 ± 0.9 [bcd]	21.5 ± 1.3 [e]	20.9 ± 1.7 [g]	1.45 ± 0.2 e	1.50 ± 0.2 [d]
VIKTORIJA	39.9 ± 1.8 [b]	38.6 ± 2.5 [c]	11.5 ± 0.9 [cd]	11.9 ± 1.4 [b]	28.4 ± 1.1 [c]	26.7 ± 1.4 [de]	1.12 ± 0.1 [i]	1.19 ± 0.1 [gh]
MV NEMERE	27.7 ± 3.5 [g]	27.8 ± 3.0 [j]	10.5 ± 1.3 [def]	11.0 ± 1.2 [def]	17.4 ± 2.4 [h]	16.8 ± 1.9 [i]	1.81 ± 0.3 [b]	1.87 ± 0.3 [b]
SOFRU	42.0 ± 4.3 [a]	40.7 ± 4.4 [a]	11.2 ± 1.5 [de]	11.8 ± 2.5 [bc]	30.8 ± 3.0 [a]	28.9 ± 3.3 [a]	0.98 ± 0.2 [k]	1.08 ± 0.2 [k]
BC LJEPOTICA	41.6 ± 3.8 [a]	39.5 ± 4.9 [b]	12.1 ± 1.7 [b]	11.3 ± 1.8 [cde]	29.4 ± 3.0 [b]	28.2 ± 3.7 [b]	1.05 ± 0.2 [jk]	1.15 ± 0.2 [ij]
MEAN	36.2 ± 5.6	35.5 ± 5.6	10.9 ± 2.0	11.0 ± 2.1	25.3 ± 4.6	24.5 ± 4.5	1.32 ± 0.4	1.38 ± 0.4
LSD$_{GEN}$ 0.05	0.9	0.8	0.6	0.6	0.7	0.6	0.06	0.05

[1] Means ± standard deviation across years and locations under low and high N levels. [2] GLU = glutenins, HMW-GS = high-molecular-weight glutenin subunits, LMW-GS = low-molecular-weight glutenin subunits, GLI/GLU = gliadin/glutenin ratio. [3] N_0 = low N level, N_{100} = high N level. [4] For each cultivar within a column, values followed by different letters are significantly different at $p < 0.05$.

The GLI/GLU ratio is related to the balance of dough strength and extensibility [32]. The negative influence of a higher GLI/GLU ratio on dough rheological properties is well known [31,32,39]. The GLI/GLU ratio under a low N level varied from 0.98 (Sofru) to 1.98 (U-1) and, under higher N supply, the GLI/GLU ratio was generally increased by 4.6% (1.32 vs. 1.38) (Table 4). The highest increase in the GLI/GLU ratio was noticed in the cultivars San Pastore, BC Ljepotica and Sofru (10.8%, 9.6% and 9.3%, respectively). In the old cultivars, the total P increase resulted in an increase in the GLI/GLU ratio (San Pastore: 1.72 vs. 1.91 and Libellula: 1.58 vs. 1.68), while the cultivar U-1 retained the highest GLI/GLU value despite its reduction by 3% under higher N (1.98 vs. 1.92). In the modern cultivars under a high N level, the GLI/GLU ratio ranged between 1.08 (Sofru) and 1.50 (Kraljica), except for cultivar MV Nemere which had a high GLI/GLU ratio at both N levels (1.81 vs. 1.87). De Santis et al. [34] reported that the better technological performance of modern Italian cultivars compared to old ones was found to be due to the presence of superior HMW-GS and LMW-GS alleles and to the differential expression of specific storage proteins and because the higher GI (55.3 vs. 9.6 for old cultivars in the first crop season and 46 vs. 7.5 in the second crop season) observed in modern genotypes was correlated with a decreased GLI/GLU ratio (1.6 vs. 2.6 for old cultivars in the first crop season and 1.8 vs. 2.9 in the second crop season). From a breeder's perspective, in order to hold their seed market share, there are presently two major concerns: to extend the target regions and to increase the end-use quality [40]. To fulfill those requirements, there is a practical consequence of the higher cost of breeding programs, due to an increased number of field trials and an increased number of laboratory tests to assess. This means that breeders are faced with the dilemma that increasing the number of trials with additional

N levels would increase their costs, but, on the other hand, there is a penalty in terms of missing information about the agronomic and quality performance and responsiveness of new genotypes. Sylvester-Bradley et al. [41] suggested that we should select for wheat cultivars showing a low N optimum and that are highly responsive to N at lower doses while, simultaneously, high N responsiveness is kept under high N conditions. In our study, N treatment had an effect on both wheat protein content and composition, but not equally on all protein fractions. The effect on GLI was more pronounced than on GLU, while AG were not as increased. Furthermore, there was a cultivar-specific reaction to N treatment. This could offer breeders a blueprint for selecting genotypes more responsive to N treatment by selecting for those protein fractions that contribute to the overall wheat quality under increased N levels, while, on the other side, selecting other protein fractions that could be more important under reduced N regimes. Ideally, such an approach could contribute to the selection of new genotypes with improved stability of quality under different soil fertility and N fertilization regimes.

4. Conclusions

Significant differences were found among analyzed wheat grain proteins depending on cultivars, nitrogen level and environment, as well as their interactions. Increasing N level enhanced grain P content and moved cultivars into a higher quality class, while the composition of quality-related GLI and GLU fractions was changed to a lesser extent and was strongly cultivar specific and therefore should be considered for wheat quality assessment and breeding purposes because higher grain protein concentrations are major targets of modern wheat breeding, together with well-balanced proportions of quality-related proteins.

Abbreviations

N	nitrogen
kg	kilogram
ha	hectare
P	protein
RP-HPLC	reversed phase–high-pressure liquid chromatography
GLI	gliadins

α-GLI	α-gliadins
γ-GLI	γ-gliadins
HMW-GS	high-molecular-weight glutenin subunits
AG	albumins/globulins
ω-GLI	ω-gliadins
GLU	glutenins
LMW-GS	low-molecular-weight glutenin subunits
GLI/GLU	gliadins/glutenins
α/β-GLI	α/β-gliadins
HMW-GS/LMW-GS	high-molecular-weight glutenin subunits/low-molecular-weight glutenin subunits
t	ton
m^2	square meter
P_2O_5	phosphorus pentoxide
K_2O	potassium oxide
NPK	nitrogen, phosphorus and potassium fertilizer
HR	Croatia
HU	Hungary
FR	France
RU	Russia
IT	Italy
GS23-25	growth stages 23–25
GS33-35	growth stages 33–35
DM	dry matter
WG	wet gluten
GI	gluten index
ICC	International Association for Cereal Science and Technology
mg	milligram
mm	millimeter
mL	milliliter
M	molar
NaCl	natrium chloride
min	minute
RT	room temperature
1-PrOH	1-propanol
v/v	volume/volume
w/v	weight/volume
Tris-HCl	Tris-hydrochloride
pH	lat. *potentia hydrogenii*
°C	degrees Celsius
rpm	rotations per minute
μm	micrometer
PVDF	polyvinylidene difluoride
HPLC	high-pressure liquid chromatography
USA	United States of America
ACN	acetonitrile
TFA	trifluoroacetic acid
H_2O	water
C18	carbon18
nm	nanometer
GEN	genotype
LOC	location
YEAR	year
HSD	honestly significant difference in Tukey's post hoc test
LSD	least significance difference
ANOVA	analysis of variance

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