



The effect of nitrogen fertilization on the expression of protein in wheat and tritordeum varieties using a proteomic approach

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ABSTRACT

Wheat, an essential ingredient for several bakery preparations, is also responsible for gluten-related diseases in sensitive subjects. The effect of the N fertilization rate (80 vs 160 kg N ha⁻¹) on gluten protein expression profile has been evaluated considering two soft wheats (landrace and modern) and one tritordeum cultivar (cv), grown in the same experimental field in North Italy. The proteins of refined flour were characterized through advanced proteomic approaches, including chromatography (RP-HPLC) and electrophoresis. A static model system was used to simulate *in vitro* digestion and the digested peptides were examined by mass spectrometry and *in silico* approaches, to investigate the celiac and allergenic sequences. The CD-toxic epitopes in the digested samples were quantified by means of a R5 ELISA assay.

The N fertilization rate increased the grain protein content, but it did not lead to any difference in gluten composition, with exception of glu/glia ratio in the modern wheat cv. Moreover, the gluten composition and the occurrence of toxic/allergenic epitopes varied to a great extent, according mostly to the genotype. A lower immunoreactivity, determined using R5 ELISA, was detected for the digested tritordeum flours than for the landrace (-51%) or modern (-58%) cvs, while no significant difference was observed for the N rates between each genotype. *In silico* analysis showed that tritordeum has fewer CD epitopes belonging to the ω -gliadins and a lower LMW-GS than the landrace or modern cv. Tritordeum presented fewer α -gliadin allergenic epitopes than the modern wheat cv. The lower frequency of celiac epitopes in tritordeum, compared to the old and the modern wheat, is probably due to the absence of a D genome.

1. Introduction

The quality of soft wheat (*Triticum aestivum* L. subsp. *aestivum*) flour influences the organoleptic and structural properties of the final products to a great extent. Modern hexaploid wheats (AABBDD) are products of breeding processes that were aimed at producing cultivars (cvs) with

an improved bread-making potential, a more balanced glutenin/gliadin (glu/glia) ratio and a higher high molecular weight glutenin (HMW-GS) / low molecular weight glutenin (LMW-GS) ratio (Dhaka & Khatkar, 2015) than wheat landraces (AABBDD) (local ecotype) or old wheat varieties, which have been cultivated since before the second half of the XX^o century (Migliorini et al., 2016). A new cereal, tritordeum (x

Abbreviations: cvs, cultivars; glu, glutenins; glia, gliadins; HMW-GS, high molecular weight glutenins; LMW-GS, low molecular weight glutenins; GS, glutenin subunits; GPC, grain protein content; CD, celiac disease; NCGS, Non-Celiac Gluten Sensitivity; CPVO, Community Plant Variety Office; TW, test weight; TKW, thousand kernel weight; NIR, Near Infrared Reflectance; ACN, acetonitrile; 1-PrOH, propan-1-ol; EtOH, ethanol; AmBic, ammonium bicarbonate; SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; TCA, trichloroacetic acid; DTT, dithiothreitol; IAA, iodoacetamide; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl) aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; TAME, *p*-toluenesulfonyl-L-arginine methyl ester; TPI, total protein isolates; 2-DE, 2-sulfanylethanol, two-dimensional electrophoresis; IPG, immobilized pH gradient; IEF, Isoelectric focusing; LIT, nano-ESI-linear ion trap; GID, gastrointestinal digested; Gln, glutamine; MW, molecular weight; OGD, oral, gastric and duodenal phases; WDEIA, wheat-dependent exercise-induced anaphylaxis.

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Triticum martini A. Pujadas, nothosp. nov.) (AABBHhCh), has recently received a great deal of scientific and commercial attention (Ribeiro et al., 2016; Vaquero et al., 2018). This amphiploid species, which is derived from the crossing of a South American wild barley (*Hordeum chilense* Roem. et Schultz.) and a durum wheat (*Triticum turgidum* L. subsp. *durum* Desf.), has been described as a good bread-making flour, with similar performances to those of soft wheat, and it has found its way into the baking industry (Martín et al., 1999).

Gluten, from a food technology perspective, is the main player in bread making, as it represents around 60% of the wheat proteome (Fiedler et al., 2014). Gluten can be subdivided, according to the Osborne classification, into 2 main fractions with the following sub-fractions: water-soluble non-prolamins, albumins and globulins; and prolamins, alcohol-soluble gliadins and polymeric alcohol-insoluble glutenins (Mamone et al., 2000). Monomeric gliadins are classified, on the basis of their electrophoretic mobility, into 3 fractions: α -, γ - and ω -gliadins. The polymeric glutenin subunits (GS) cover a wide range of molecular weights, and are conventionally classified as LMW-GS (12–60 kDa) or HMW-GS (up to 120 kDa) (Mamone et al., 2000). The rheological properties of the flour depend on the quality and the quantity of the gluten and in particular on the glu/glia and HMW-GS/LMW-GS ratios. A large amount of gliadins increases the viscosity of a dough, thereby influencing its extensibility, while a greater quantity of glutenins enhances the strength of the dough, thereby influencing its elasticity (Plessis et al., 2013). Gluten strength is defined by considering the composition of the gluten proteins, the quantity of the HMW glutenin subunits and the glu/glia ratio (Johansson et al., 2001).

The meteorological and soil conditions, the type of cv and the different nitrogen rates are all factors that qualitatively and quantitatively modulate the flour protein profile (Godfrey et al., 2010). The environmental conditions can influence the grain protein content (GPC) to a greater extent than the cv (Wan et al., 2013). Moreover, N fertilization is a crop technique that is expected to quantitatively change the GPC and the rheological properties of the derived flour (Garrido-Lestache et al., 2004).

The mapping of gluten expressed proteins is fundamental to assess the technological quality of different cereal species and cvs (Cho et al., 2018). Gluten proteins can cause inflammation of the small intestine in subjects affected by celiac disease (CD), which is a common autoimmune enteropathy of the small intestine, whose prevalence has risen in the last 50 years and today affects about 1% of Western populations (Lebwohl et al., 2015). The pathogenesis of CD begins with the gluten digestion derived peptides, which are transported throughout the microvilli and trigger an immune response of the T-cells (Dunne et al., 2020). CD disease has multifactorial etiologies, including the interaction of genetic factors in susceptible individuals (HLA-DQ2 or -DQ8 haplotype), environmental components and immunological mechanisms (Kagnoff, 2007). Non-Celiac Gluten Sensitivity (NCGS) is a “new” condition that has emerged in recent years, and it affects up to 6% of the world’s population (Casella et al., 2018). Its diagnosis is a challenging task as it requires the clinical exclusion of CD and wheat allergies, and the symptoms are hard to distinguish from those of the irritable bowel syndrome (Casella et al., 2018). The triggering causes are not clear and require further investigation. Triticum bread was recently found to be tolerated by subjects with non-coeliac wheat sensitivity (NCWS) to a similar extent to gluten free bread (Sánchez-León et al., 2021). Extensive characterization studies, including proteomics and immunobased approaches, aimed at identifying the epitopes and toxic motifs responsible for triggering allergic and toxic reactions, are necessary. An extensive collection of known epitopes is available in the ProPepper™ database (Juhász et al., 2015).

A deep understanding of the role of the environmental and agronomical conditions, specifically the choice of cv and N fertilization, on the gluten composition and expression of specific epitopes, could provide useful technological information for bread making. In this respect, the type of selected wheat cv can also make a great difference in the

consumers’ acceptance of bakery products. In this paper, we present a proteomic characterization of three types of cereal grains: landrace wheat, modern wheat and tritordeum. Proteins isolated from grain flours were profiled using advanced proteomic approaches, including chromatography and electrophoresis. The flours were digested using an in vitro batch model to simulate the oral, gastric and duodenal compartments of humans. Mass spectrometry was used to create a picture of the obtained digestome. The collected peptide sequences were analyzed *in silico* for the presence of toxic and allergenic sequences. Additionally, a competitive ELISA analysis was performed on the digestome to evaluate any diversification of the immunoresponse related to fertilization and to the genotype.

2. Materials and methods

2.1. Experimental site and treatments

The effect of the N fertilization rate was evaluated by means of a full factorial experimental design, considering 2 soft wheat and 1 tritordeum cvs, characterized by different gluten compositions.

Two N fertilization treatments were compared: a low N rate (80 kg N ha⁻¹, N80) and a high N rate (160 kg N ha⁻¹, N160). The total N rate for each treatment was top-dressed applied as a granular ammonium nitrate fertilizer, split equally between tillering (growth stage 23) and the beginning of stem elongation (growth stage 32).

The considered genotypes were:

- Landrace, cv Andriolo, which was mainly cultivated in mountain areas in the Tuscany region (Italy) in the XIXth century, and is a soft wheat cv (hexaploid AABBDD) (Migliorini et al., 2016);
- Modern, cv Bologna (S.I.S., San Lazzaro di Savena, Bologna, Italy; genealogy (H89092 X H89136) X Soissons), is a soft wheat (hexaploid AABBDD) cv, classified as improver wheat according to the Synthetic Index of Quality (Foca et al., 2007), which was registered in the Italian varietal list in 2002 (<https://www.sian.it/mivmPubb/listeVarieta.do>);
- Tritordeum, cv Bulel (Agrasys S.L., Barcelona, Spain), which was registered in the CPVO List (Community Plant Variety Office) in 2011 (hexaploid AABBHhCh).

A field study was carried out in the Po plain at Carmagnola in North West Italy (44° 50' N, 7° 40' E; elevation 245 m), in the 2016–17 growing season. The experiment was performed on the University of Turin experimental farm in a deep silty-loam soil (Typic Udifluvents), characterized by a medium cation-exchange capacity and organic matter content.

The same agronomic techniques were adopted for all the cvs. Briefly, the previous crop was soft wheat, and planting was performed in 12 cm wide rows at a seeding rate of 400 seeds/m² on 27 October 2016, following an autumn plowing (30 cm) and disk harrowing to prepare a proper seedbed. The weed control was in accordance with the agronomic management practices usually carried out in the North of Italy for the cultivation of soft wheat, while no fungicide or insecticide was applied. Harvesting was carried out with a combine-harvester on 13 July 2017. The N rates (in three replicates) were assigned to experimental units, using a completely randomized block design. The plot size was 7 × 1.5 m.

2.2. Grain yield and kernel quality traits

The grain yields were obtained by harvesting the whole plot with a Walter Wintersteiger cereal plot combine-harvester. Grain moisture was analyzed using a Dickey-John GAC2100 grain analyzer (Auburn, IL, The USA). The grain yield results were adjusted to a 13% moisture content. Three kg grain samples, which were milled (Bona 4RB mill) to obtain refined flour, were obtained from each plot.

The test weight (TW), thousand kernel weight (TKW) and grain protein content (GPC; $N \times 5.7$, on a dry matter basis) were determined according to Blandino et al. (2015). GPC was determined on whole grains (1-mm-sieve Cyclotec mill), by means of NIR (Near Infrared Reflectance) spectroscopy, according to AACC 39–10 (AACC, 2000).

2.3. Materials and chemical reagents

All the reagents used in this study were of analytical or higher grade. The HPLC-grade solvents: water, acetonitrile (ACN), propan-1-ol (1-PrOH) and ethanol (EtOH) were from Carlo Erba (Milan, Italy). Sodium phosphate, ammonium bicarbonate (AmBic), acetic acid and the other chemicals, used to produce the simulated salivary fluid (SSF), the simulated gastric fluid (SGF) and the simulated intestinal fluid (SIF), were also provided by Carlo Erba. The enzymes used for in vitro human digestion were purchased from Sigma (St Louis, MO, USA), in line with those indicated by the Infogest protocol (Brodkorb et al., 2019). Trichloroacetic acid (TCA), dithiothreitol (DTT), iodoacetamide (IAA), sodium dodecyl sulfate (SDS), glycerol, tris(hydroxymethyl) amino-methane hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), guanidine chloride, trifluoroacetic acid (TFA), 2-vinylpyridine monomer and *p*-toluenesulfonyl-L-arginine methyl ester (TAME) were purchased from Sigma-Aldrich (St Louis, MO, USA). Egg lecithin was purchased from Lipid Products (Redhill, UK). The electrophoresis reagents were all from Bio-Rad (Milan, Italy).

2.4. Protein extraction

2.4.1. Production of the total protein isolate

The total protein isolate (TPI) of the wheat samples was produced according to the procedure of (Dupont et al., 2011). An aliquot of 1 ml of SDS buffer (2% SDS, 10% glycerol, 50 mM DTT, 40 mM Tris-HCl, pH 6.8) was added to the flour samples (50 mg), which were then incubated at room temperature with discontinuous mixing for 1 h. The obtained pellets were removed by centrifugation at 7,900 g for 10 min (Micro-centrifuge Multispin 12, Steroglass, Perugia, Italy). The proteins in the supernatant were precipitated with 4 vol cold (-20°C) propan-2-one, following incubation overnight at -20°C and centrifugation at 7,900 g for 10 min. The pellets were dried under a nitrogen stream and stored at -20°C .

2.4.2. Selective extraction of the gliadins and glutenins

The protein fractions were extracted according to Mamone et al. (2000), with slight modifications. The albumins and globulins were removed from non-defatted flour (1 g) by adding a solution (10 ml) containing 100 mM KCl, 50 mM Tris-HCl pH 7.8 and 5 mM EDTA, and centrifuging for 15 min at 3,433 g. After removal of the supernatant, the gliadins were extracted with 10 ml of 70% (v/v) ethanol (twice) and the glutenins with 50% v/v 1-PrOH; 50 mM Tris-HCl (pH 8.5); 1% (w/v) DTT. Glutenin extraction was performed at 60°C for 30 min. The solutions were mixed for 2 min and stirred on a magnetic plate for 10 min. The cysteine residues of the glutenin extracts were pyridylethylated (VP glutenins) at 60°C for 15 min with 2-vinylpyridine.

2.5. HPLC analysis

Reversed phase (RP)-HPLC analysis of the gliadins and glutenins was carried out in an HPLC chromatograph (HP 1100 Agilent, Palo Alto, CA, USA) modular system equipped with a diode array detector. The column effluent was monitored, by means of UV detection, at $\lambda = 220$ and 280 nm. The separation was performed using a C8 Vydac, 208TP52, 2.1×250 mm, $5 \mu\text{m}$ column (Hesperia, CA, The USA). Solvent A was 0.1% TFA v/v in water, while solvent B was 0.1% TFA in ACN. After 5 min of isocratic elution, using 25% solvent B (0.1% TFA in ACN, v/v), a 25–60% gradient ramp was applied for 60 min at a flow rate of 0.200 ml/min. This procedure was performed according to the work of

Mamone et al. (2000) with some modifications. The peaks were then integrated using Agilent ChemStation software to obtain the relative quantity of both the gliadins and glutenins in each sample.

2.6. SDS-PAGE of the gliadins and glutenins

The glutenins and gliadins obtained from the selective extraction (section 2.4.2.), were precipitated in cold propan-2-one (1:4 v:v), suspended in an SDS-PAGE Laemmli Buffer [0.125 M Tris-HCl pH 6.8, 5% SDS, 20% glycerol, 5% (w/v) 2-sulfanylethanol, 0.02% bromophenol blue and boiled in a water bath for 5 min. The proteins (10 mg/ml) were loaded onto a 12% polyacrylamide gel in a final volume of 3 μL . Analysis was carried out at room temperature and constant voltage (100 V). After migration, the proteins were fixed overnight with TCA (24%) and stained with Coomassie G-250. The electrophoretic profiles were analyzed using GelAnalyzer 19.1 software (www.gelanalyzer.com by Istvan Lazar Jr., PhD and Istvan Lazar Sr., PhD, CSc).

2.7. Two-dimensional electrophoresis analysis of the total protein isolate (TPI)

The TPI was dissolved (3 mg/ml) in an immobilized pH gradient (IPG) strip rehydration buffer, containing 8 M urea, 2% (w/v) CHAPS, 2% (v/v) Pharmalytes pH 3.0–10.0, 20 mM DTT and traces of bromophenol blue, for two-dimensional electrophoresis (2-DE). The isoelectric focalisation was performed using pH 3–10 Immobiline Dry Strips (11 cm) linear gradient, from Bio-Rad Laboratories. The strips were rehydrated overnight in an Immobiline Dry-Strip Reswelling Tray (Amersham Pharmacia). Isoelectric focusing (IEF) was carried out using the Multiphor II system (Pharmacia Biotech, Uppsala, Sweden). The proteins were focused on up to 15,000 Vh at a maximum voltage of 6000 V at 20°C . After focusing, the proteins were reduced for 15 min in an equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 2% DTT), and alkylated for 15 min with 2.5% IAA. SDS-PAGE was performed in the second dimension on a 10% polyacrylamide gel, using a Protean II system (BioRad Laboratories, Hercules, California, USA). The run was performed at a constant voltage of 220 V. After migration, the proteins were fixed overnight with TCA (24%) and stained with Coomassie G-250.

2.8. In batch gastroduodenal digestion of flour

Flour samples were subjected to in vitro simulated human digestion, using the static model system optimized in the framework of the Infogest COST Action project (Brodkorb et al., 2019). Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the harmonized conditions (Brodkorb et al., 2019). Mastication was simulated using a manual mincer. One g of flour was mixed with 1 ml of SSF and minced for 2 min at 37°C . The SSF included 1500 U ml^{-1} human salivary amylase. SGF was added to the bolus (50:50 v/v); a concentration of up to 0.17 mmol/L of gastric liposome (egg lecithin prepared in a vesicular form) and 2000 U/ml of SGF porcine pepsin was added to this mixture. The pH of the digesta was adjusted to 2.7 and the incubation was carried out for 2 h at 37°C under magnetic stirring. After adding SIF (50:50 v/v) to the gastric digesta, the pH was adjusted to 7.0, using NaOH 1 M. Ten mmol/L of bile salts (5 mmol/L sodium taurocholate, 5 mmol/L sodium glycodeoxycholate and 1.8 mmol/L egg lecithin in the final concentration), and pancreatin were added to the intestinal mixture. The amount of pancreatin was calculated on the basis of the measured trypsin activity, and a final volume of up to 100 U of trypsin/ml was added. Trypsin activity was determined by means of a TAME assay (Brodkorb et al., 2019). Each sample was incubated for 2 h at 37°C under magnetic stirring. The enzymatic activity was stopped by boiling the samples for 5 min in a water bath. The samples were then centrifuged at 3,433 g for 15 min to collect the supernatants, which were then stored at -20°C until use. The digestion

was monitored by means of SDS-PAGE analysis. A part of the supernatant of each sample (1 ml) was desalted using a Phenomenex (Torrance, CA, USA) Strata-X SPE cartridge following the manufacturer instruction and then concentrated with gaseous nitrogen before the LC-MS/MS analysis was performed.

2.9. R5 competitive ELISA assay on the digested samples

RIDASCREEN® Gliadin competitive (Art. No. R7021, R-BIOPHARM AG, Darmstadt, Germany) was used according to the manufacturer's instructions. This Competitive ELISA relies on the use of the R5 monoclonal antibody (Di Stasio et al., 2020). The gastroduodenal digestion products were analyzed in duplicate.

2.10. LC-MS/MS analysis

LC-MS analysis was performed using a Dionex UltiMate 3000 nano-UHPLC system coupled with a nano-ESI-linear ion trap (LIT) Thermo XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, The USA). Samples were resuspended in a 0.1% (v/v) formic acid solution, loaded through a 5 mm long, 300 µm id pre-column (LC Packings, The USA) and separated in an Acclaim™ PepMap™ C18 column (150 mm × 75 µm, 3 µm) at a flow rate of 0.200 µL/min. Eluent A was 0.1% formic acid (v/v) in Milli-Q water; eluent B was 0.1% formic acid (v/v) in ACN. The column was equilibrated at 5% B. Peptides were separated by applying a 5–40% gradient of B over 40 min. MS data were obtained over the 200 to 2000 *m/z* mass range. Data-dependent MS/MS spectra were collected from the five most abundant precursor ions upon fragmentation (charge state ≥ 2; isolated width: 2 Da; min. signal required:

500), using CID activation with 35.0% normalized collision energy, an activation Q of 0.25, and an activation time of 30 ms. The spectra were processed using Xcalibur Software, 3.1 version (Thermo Scientific). The mass spectra were then analyzed using Protein Prospector software. The GluPro v 1.2 database of wheat gluten protein sequences (Daly et al., 2020) was used as background database for the analysis of the mass spectrometry data. The database searching parameters used for the identification of the peptidomes of the simulated gastrointestinal digested (GID) flour included “no enzyme” specification, and Met-oxidation and pyroglutamic acid for N-terminus glutamine (Gln) as variable protein modifications, with a mass tolerance value of 1 Da for the precursor ion and 0.6 Da for the MS/MS fragments. Analyses were carried out in triplicate.

2.11. Searching criteria for the celiac disease related epitopes, allergic epitopes and biopeptides

The identified peptides were searched manually for the presence of described toxic (CD epitopes), allergenic (IgE-binding epitopes) and bioactive amino acid sequences. The applied bioinformatic pipeline is described in Fig. 1. The ProPepper database (Juhász et al., 2015) and the CD epitope table presented in Mamone et al. (2011) were used as the sources of the CD-toxic motifs. Only toxic motifs that appeared in at least 3 identified peptides were selected for comparison.

The IgE binding sequences were retrieved from the Immune Epitope Database (IEDB) (Vita et al., 2019), a repository of peer-reviewed epitopic sequences that have shown proved ability to trigger immune responses. The following search parameters were applied to extract IgE epitopes from the database: substring, allergy disease, and Triticum: ID

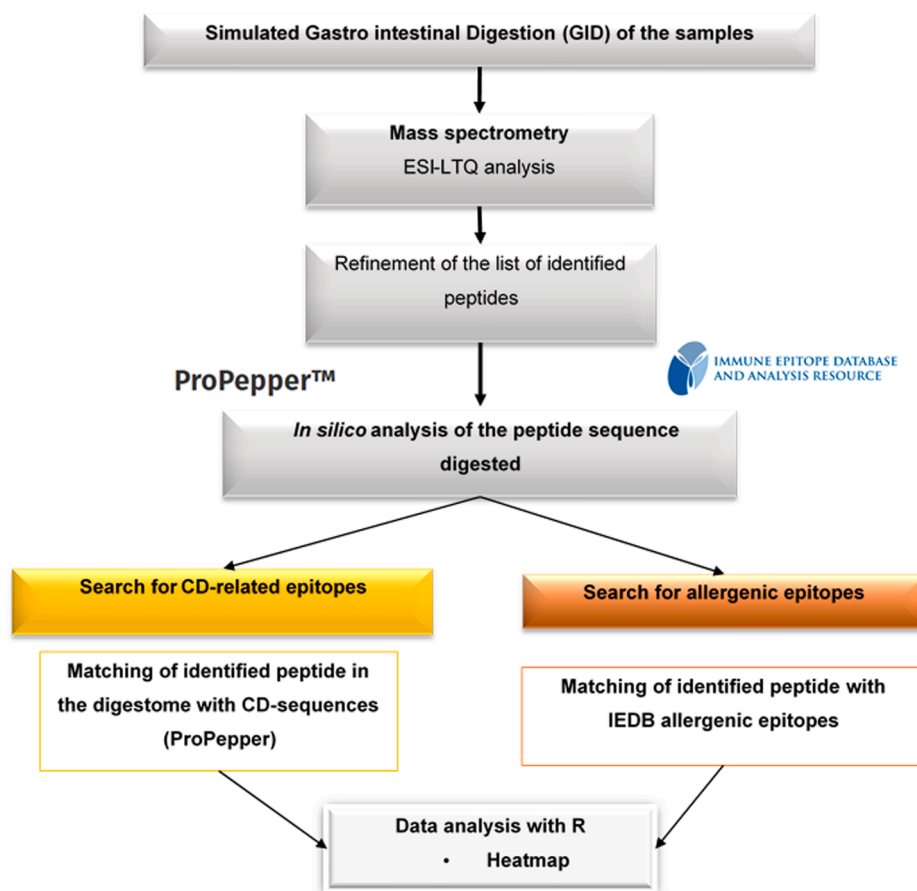


Fig. 1. Flow chart of the in silico analysis after in vitro digestion and Mass spectrometry analysis (ESI-LTQ). The identification of the CD and allergen epitopes obtained thanks to the already indexed ones. *Peptides were defined unique within the given database. Uniqueness was assessed using Skyline software and matching the list of identified peptides with the reference database as background in the “import peptide list” window.

4564. RStudio® (64-Bit-R-3.6.3) was used to create a heatmap to represent the density of the verified epitopes per cv/N rate. The use of grayscale heatmaps permitted the distribution of the analyzed samples of the CD and IgE-binding epitopes to be visualized.

The bioactive peptides were retrieved from a literature review (Babini et al., 2017; Liu & Udenigwe, 2019; Suetsuna & Chen, 2002).

2.12. Statistical analysis

The agronomic parameters (grain yield, TW, TKW), the GPC, the glu/glia and HMW/LMW-GS ratios and the percentage of single gliadin and glutenin fractions were compared by means of an analysis of variance (ANOVA), in which the combination of genotypes and N fertilization were the independent variables. Multiple comparison tests were performed, according to the Ryan-Einot-Gabriel-Welsh F (REGW-F) test, on the treatment means. Statistical data analysis was carried out with the SPSS software package, version 26.0.

3. Result and discussion

3.1. Agronomical and productive parameters

Due to the good soil fertility of the site where the experiment was carried out, the N rate did not result in any significant increase in grain yield, TW or TKW for any of the compared cvs (Table 1). Clear differences were observed in the productive parameters and grain traits of the compared cvs. The grain yield of modern cv was 57% and 33% higher than that of landrace and tritordeum, respectively. Modern wheat showed the highest test weight value and the lowest TKW, the opposite of the landrace cv, which showed the highest TKW and lower values of TW, as a consequence of strong lodging under both N fertilizations.

3.2. Protein content

Although in the considered productive conditions, N fertilization did not have a significant impact on the grain yield, the GPC was clearly influenced by the combination of cv and the N application (Table 1).

On average, the highest GPC was recorded for landrace (17.7%) and tritordeum (16.6%), followed by the modern wheat cv (14.9%). A significant increase in the protein content was recorded in the landrace (+1.1%) and tritordeum (+1.5%) cv kernels for with a double N rate. The GPC increased in N160 for the modern wheat, albeit not significantly (+0.7%). This increase confirms previous results in literature (Garrido-Lestache et al., 2004; Godfrey et al., 2010; Johansson et al., 2001). To better understand the nutritional value of tritordeum, it will be interesting in future works to verify the increase in essential amino acids related to the N fertilization (Zhang et al., 2016).

Table 1

Effect of the cultivar and N rate on the grain yield, test weight (TW), thousand kernel weight (TKW) and grain protein content (GPC).

Cultivar	N rate	Grain yield	TW	TKW	GPC
	(kg N/ha)	(t ha ⁻¹)	(kg hl ⁻¹)	(g)	(%)
landrace	N80	2.9 c	73.8 b	45.0 a	17.1 b
	N160	3.0 c	74.5 b	45.6 a	18.2 a
modern	N80	6.8 a	77.3 a	33.0 c	14.5 d
	N160	7.0 a	76.2 a	31.9 c	15.2 cd
tritordeum	N80	4.8 b	70.3 c	36.8 b	15.8 c
	N160	4.2 b	69.4 c	36.1 b	17.3 b
p-value		< 0.001	< 0.001	< 0.001	< 0.001

Means followed by different letters are significantly different (the level of significance, p-value, is reported in the table), according to the REGW-F test.

3.3. Proteomic analysis of the whole grain protein and gluten fractions

3.3.1. DE of the whole grain proteins

The 2-DE separation of crude extracted proteins for all the cvs/N rates allowed maps to be generated to show the typical migration patterns of the main cereal protein families (Fig. S1). Distinctive spots were observed for each subfraction with corresponding molecular weight (MW) ranges of 80–120 kDa for HMW-GS, 60–68 kDa for the ω5-gliadins, 43–60 kDa for the ω1,2-gliadins and 32–45 kDa for the α- and γ-gliadins and LMW-GS (Mamone et al., 2005).

The analysis revealed differences in the protein expression across the considered cvs but did not show macroscopic qualitative differences that could be attributed to the N rates. Tritordeum flour appeared to have the proteome with the largest qualitative differences in the presence of protein spots in the acid region around 31 kDa, corresponding to the LMW glutenins and the gliadin region (α, γ). These differences were also clear in the SDS-PAGE profile of the isolated gliadin fraction (Fig. S2) and can be attributed to the nature of the tritordeum cv, which is an amphiploid of *T. turgidum* and *H. chilense* (Alvarez et al., 1995, 1999; Sillero et al., 1999).

3.3.2. SDS-PAGE and analysis of the osborn fractions

The gliadin and glutenin Osborne extracts were separated by means of SDS-PAGE. The glutenins (Fig. S2_B) showed the typical separation of the HMW-GS (80–140 kDa) and LMW-GS (10–70 kDa) families. The glutenin expression in the analyzed cvs clearly differed. The gel analysis highlighted 8 bands in the landrace samples, while only 6 bands were observed in the modern wheat and tritordeum. Unlike the modern and landrace samples, tritordeum presented a less complex profile in the HMW glutenin region. This observation is in line with the calculation of HMW/LMW ratios retrieved from the HPLC profile (Table 2). The tritordeum gliadins showed a more complex profile, which results in a higher number of bands than the modern and landrace cvs (Fig. S2_A). Interestingly no qualitative diversification, as induced by the N effect, was observed among the samples.

3.3.3. HPLC analysis

The RP-HPLC gradient separation of the gliadin and glutenin fractions was optimized on a RP C8 column to achieve high resolution profiles. The chromatographic profile of the gliadins (Fig. S3) showed the typical peaks attributed to ω5, ω1,2, α- and γ-sub fractions, and allowed the relative amounts of the expressed gliadin subfractions to be determined (Table 2) (Mamone et al., 2000). In all the genotypes, the α- and γ-fractions had similar values and were more abundant than the ω-gliadins. Both cv and N rate factors did not influence the relative amount of α- and γ-gliadins fractions. Also Vaquero et al. (2018) reported a stable behaviour of these gliadin fractions across different tritordeum and wheat genotypes. Conversely, the ω5 fraction was significantly higher in the wheat landrace compared to the tritordeum, while the modern cv had an intermediate content. This evidence is interesting, considering the potential IgE-binding capacity associated with this gliadin fraction (Matsuo et al., 2004).

The same approach was used to analyze the glutenin isolated fraction, and the determined HMW/LMW-GS ratios for each cv/N rate are reported in Table 2. Unlike the gliadin fraction, the glutenin fraction in tritordeum differed significantly from the other two wheat cvs for both glutenin subunits, leading to a HMW/LMW-GS ratio significantly lower than both the landrace and modern wheat cvs.

As far as the gluten fraction composition, the N rate significantly influenced only the glu/glia ratio in the modern wheat cv. Otherwise, N fertilization did not affect the prolamin ratio in landrace and tritordeum cvs, confirming results of Johansson et al. (2001). For these cvs, it is supposed that the higher N rate could have increase to a similar extent all the single individual storage protein components (Zhen et al., 2020), and they have equally contributed to the rise of GPC (Martre et al., 2003).

Table 2

Effect of the cultivar and N rate on the glutenin/gliadin (glu/glia) and HMW/LMW-GS ratios and the percentage of the single fractions of gliadin and glutenin, as obtained from the HPLC-MS analysis.

Cultivars	N rate (kg N/ha)	glu/glia	HMW/LMW-GS	Gliadins			Glutenins		
				ω 5	ω 1,2	α	γ	HMW-GS	LMW-GS
landrace	N80	0.84 b	0.70 a	2.8 a	5.8 a	43.9 a	47.5 a	41.2 a	58.8 b
	N160	0.88 b	0.83 a	2.7 a	4.2 a	45.4 a	47.7 a	45.5 a	54.5 b
modern	N80	0.89 b	0.79 a	1.4 bc	5.8 a	44.7 a	48.1 a	44.1 a	55.9 b
	N160	1.16 a	0.82 a	2.1 ab	5.5 a	42.3 a	50.1 a	45.0 a	55.0 b
tritordeum	N80	0.96 b	0.29 b	1.1 c	4.3 a	53.4 a	41.2 a	22.6 b	77.4 a
	N160	0.99 b	0.30 b	0.9 c	5.5 a	52.3 a	41.3 a	22.9 b	77.1 a
p-value		0.001	<0.001	0.001	0.251	0.119	0.063	<0.001	0.002

Means followed by different letters are significantly different (the level of significance, *p*-value, is reported in the table), according to the REGW-F test.

3.4. Residual immunoreactivity of the digested flours by means of ELISA

The immunoreactivity of the peptides generated during the gastrointestinal digestion of flours was tested using a competitive enzyme-linked immunosorbent assay (Di Stasio et al., 2020). The assay uses a monoclonal antibody to target the “QQPFP” repeated sequence found in prolamins.

Digested tritordeum flour showed a lower immunoreactivity than that of the landrace (-50%) and modern (-58%) cvs. No significant differences were identified for the samples with different N rates for any of the cvs. The lower expression of the above-mentioned celiac-toxic epitopes in tritordeum, which could be attributed to the absence of genome D, is shown in Fig. 2.

This evidence prompted us to conduct a more detailed structural analysis of the protein gastrointestinal digests of the flour by means of mass spectrometry.

3.5. Mass spectrometry profiling of the gastrointestinal digested flours

In CD, the peptides derived from the digestion of food transmigrate through the intestinal epithelium. They are then deamidated in the subepithelial lamina propria by the tissue transglutaminase and presented to the T-cells. An already validated static and multi-phasic *in vitro* digestion model (Brodkorb et al., 2019), comprising oral, gastric and duodenal sequentially simulated phases (OGD), was applied to collect sequence level information on the peptides resistant to OGD proteolysis. The mass spectrometry analysis allowed several flour derived peptides to be identified which indicated a resistance to digestive enzymes, as already described in literature (Mamone et al., 2015). The natural presence of protease and amylase inhibitors in the grain

flours may have contributed to the reduced digestibility. However, being pepsin the first proteolytic enzyme in the stomach environment where the pH is below 3.0, their inactivation is possible. The analysis of metabolic proteins has not been objective of the study and may require a dedicated investigation.

The main peptides that were identified belonged to the digestive enzymes that were undergoing a natural and expected autolysis (Mamone et al., 2015). The grain derived identified peptides are listed in tables S1-6. The peptides were inferred to a single protein or to protein families when no unique peptide could be identified. We identified CD peptide sequences, derived from the γ -hordein and B1-hordein fractions, in the digestome of the tritordeum flour. Although the *p*-value of the identified peptides, which reflects the probability of finding a random peptide, was acceptable (*p*-value < 0.05), the high number of repeated regions and the high homology sequences, which are typical of wheat gluten proteins, led to high E-values, a parameter that describes the number of hits one can “expect” to see by chance when searching a database of a particular size. The E-value is obtained by multiplying the *p*-value by the total number of qualified peptides in the searched database, the masses of which fall into the precursor ion mass range, plus/minus the specified tolerance (Alves & Yu, 2015). Since the LTQ mass spectrometer works at a low resolution, a tolerance of 1 Da was applied, and this caused the E value to be large. The peptides were validated manually, to confirm the software identifications, and exemplar spectra are provided in the supplementary material section (Fig. S4).

3.6. *In silico* evaluation of the *in vitro* gastrointestinal identified peptides

The digestion-derived peptides were *in silico* assessed to evaluate sequences known to potentially trigger CD, and allergic reactions. We also evaluated positive sequences with potential bioactivity.

3.7. Celiac toxic motifs

The toxic motifs of CD predominantly arise from α -gliadins (Ozuna et al., 2015), which were found to be the most abundant gliadin fraction in our experiment (about 60%), followed by γ -type gliadins (Fig. 3).

The same number of epitopes belonging to the α -gliadins was observed for modern and tritordeum, while landrace showed 15% more. Landrace also showed the highest numbers of identified epitopes for the γ -gliadins, followed by tritordeum (-18%) and then modern (-27%). Tritordeum showed 50% fewer CD epitopes belonging to the ω -gliadins and 23% and 44% lower epitope numbers of the LMW-GS class than the landrace and modern cvs.

The greatest number of CD epitopes was found in the α -gliadin class in all three varieties. On the basis of our observations, it can be seen that the most frequently identified toxic motifs (*i.e.* IPEQ, PQQLPQ, QPQQPF, QPQFPF and VRVPVPQL) are similar to those identified in previous studies and they were identified in all the studied samples (Fig. S5) (Osman et al., 2000). For example, the presence of the CD epitope IPEQ was observed in all the digested flours, and it has already been described in the consensus sequences recognized by IgG human

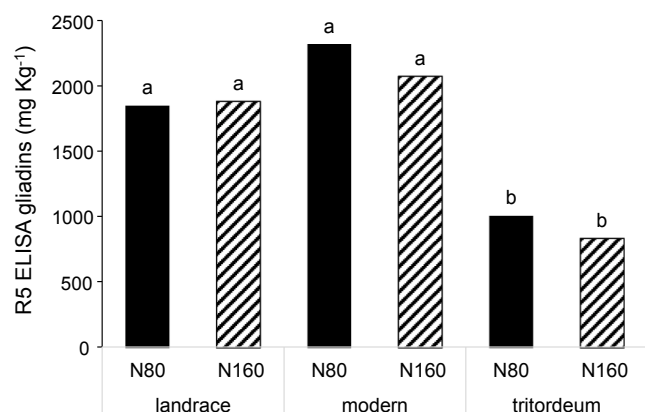


Fig. 2. Quantification of the “QQPFP” celiac toxic motif recognized by the R5 monoclonal antibody in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha⁻¹) and N160 (160 kg N ha⁻¹) fertilization rates. Bars with different letters are significantly different (*p*-value < 0.05), according to the REGW-F test.

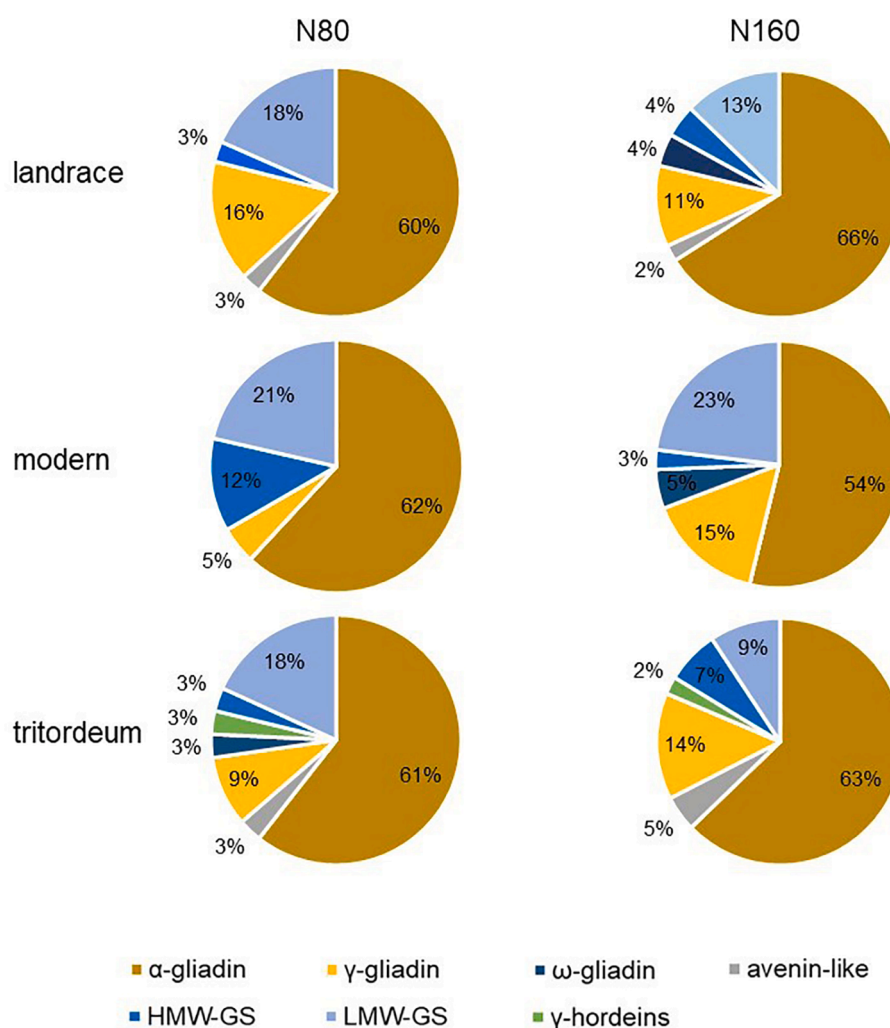


Fig. 3. Gluten sources of the celiac epitopes in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha⁻¹) and N160 (160 kg N ha⁻¹) fertilization rates.

antibodies in a Pepscan experiment (Osman et al., 2000). Similarly, QQQPFP and PQQLPQ, which were also identified in all the flours, were recognized by human IgG and IgA antibodies. The QGSFQP sequence was only identified in landrace and modern at a high N rate and in both the tritordeum samples (Osman et al., 2000). Truncated versions of the 33-mer (GliA-α 57–89, LQLQFPQPQLPYPQPQLPYPQPQLPYPQPQPF) and the 25-mer (GliA-α 31–55, LGQQQFPFPQPYPQPQFPSPQPY) gliadin derived peptides, which have been described as being resistant to digestion, and are known to be strong stimulators of the T-cell response, were identified in all the analyzed samples (Table S7) (Ozuna et al., 2015). The full length 33-mer peptide contains three repeated regions (p62–67, PQQLPY) and is exclusively present in a D-genome encoded α-gliadin (Camarca et al., 2009). The D-genome in tritordeum is replaced by the Hch genome of *Hordeum chilense*. A single fragment of 33-mer (LQFPQPQLPYPQPH) was unexpectedly identified in the tritordeum digested flour. Although the breeding program developed crosses between soft wheat and tritordeum in order to improve its yield and bread-making quality (Ávila et al., 2021), and a small introgressions from D genome into tritordeum cannot be ruled out, our experiment was carried out using a tritordeum cv carefully selected for the absence of any soft wheat chromosomes. However, the *Hordeum chilense* genome has not yet been sequenced, and the natural presence of the peptide in the tritordeum proteome cannot therefore be excluded. Alternatively, a cross-contamination might also have occurred. A targeted approach would be required to confirm the presence of partial 33-mer-like

sequences in tritordeum.

3.8. Allergenic epitopes

Wheat-dependent, exercise-induced anaphylaxis (WDEIA) is a serious allergy in which the combination of wheat ingestion and physical exercise leads to anaphylaxis. Patients with WDEIA have IgE antibodies against ω5-gliadin, one of the major gluten allergens (Lehto et al., 2003). Together with the evaluation of the CD sequences, the presence of IgE-binding sequences was also evaluated (Vita et al., 2019). The IEDB epitopic sequence was manually searched in the pool of digested peptides. An epitope was considered as present when at least one precursor peptide was identified in the digestome. In order to compare the samples, we only took into consideration the presence of the epitopic sequence, and not the number of times the sequence was identified. A graphic example of the search for GID sequences with a known allergen IEDB sequence is shown in supplementary Table S8.

The digestive derived peptides were only considered for a comparative evaluation when 100% sequence homology was shared with the described IgE binding epitope. The relative abundance of the identified epitopes (Fig. S6) was used to qualitatively compare the different cultivars and N rates. The largest number of allergenic epitopes belonged to the γ-gliadin fraction (about 40%), followed by epitopes of HMW-GS and α-gliadins prolamins for all the digested samples (Fig. 4). The modern cv was distinguished by a greater percentage (30%) of α-gliadin allergenic

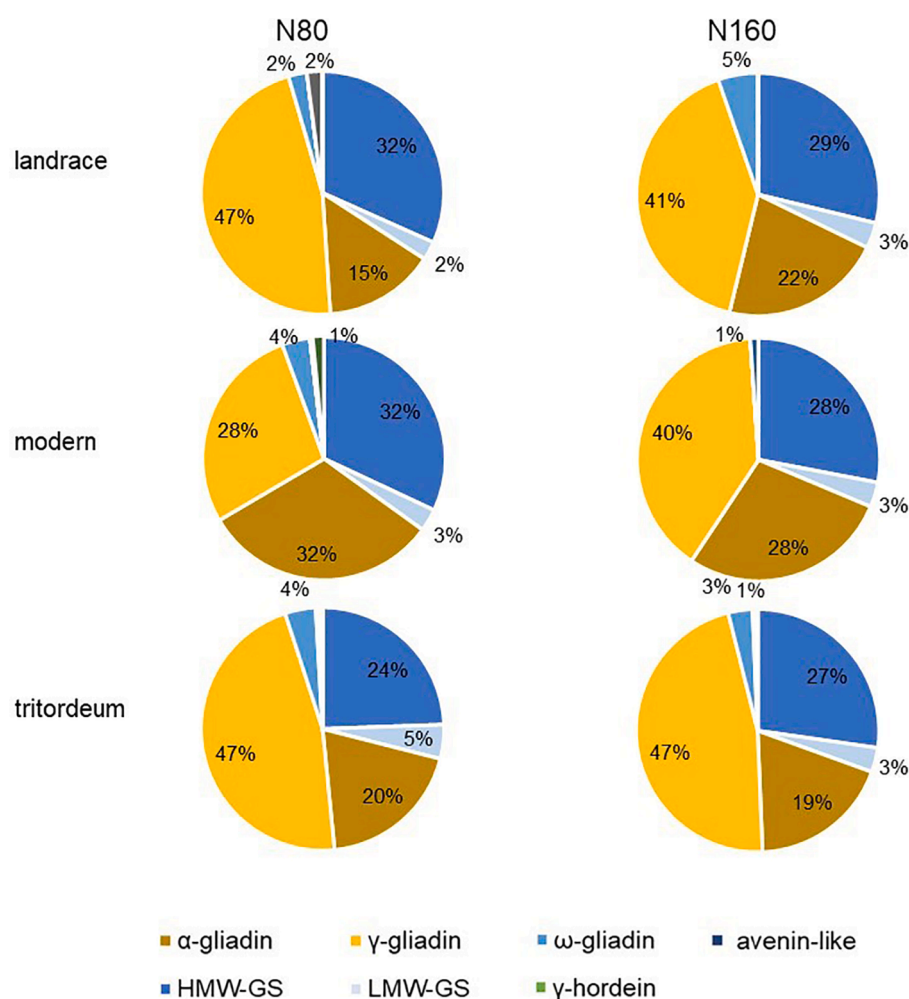


Fig. 4. Gluten sources of the allergenic epitopes in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha⁻¹) and N160 (160 kg N ha⁻¹) fertilization rates.

epitopes, but showed a lower percentage for HMW-GS, LMW-GS and the ω-gliadins than landrace and tritordeum.

A limited number of the identified epitope sequences which belong to the 5 main gluten allergens, according to the World Health

Organization and the International Union of Immunological Societies (WHO/IUIS), were identified in all the cvs and at both N rates. This allergenic wheat gluten proteins list includes (<http://www.allergen.org>): Tri a 19 (ω5), Tri a 20 (γ), Tri a 21 (α), Tri a 26 (HMW-GS) and

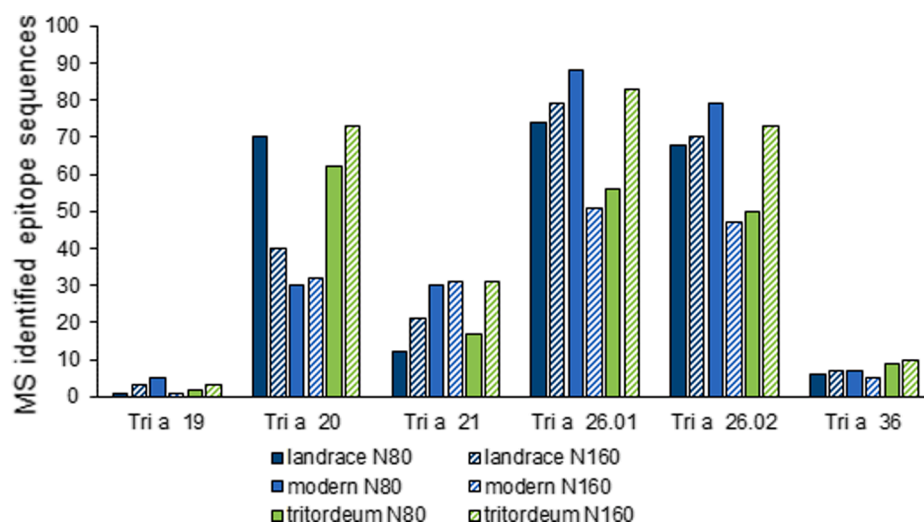


Fig. 5. A graphical qualitative representation of the MS identified epitope sequences inferred to the five main 5 main wheat allergens (tri a 19, tri a 20, tri a 21; tri a 26.01; tri a 26.02; tri a 36) in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha⁻¹) and N160 (160 kg N ha⁻¹) fertilization rates.

Tri a 36 (LMW-GS) (Fig. 5). Tri a 26 (HMW-GS) was the protein with the highest number of identified epitope sequences in all the cv and N rate combinations. HMW-GS presents disulfide-bridge linked aggregates, which induce a high resistance to in vitro enzymatic digestion (Anderson et al., 1984).

The second allergen with the highest number of epitope sequences identified was Tri a 20, belonging to the γ -gliadins, which has been identified as a strong trigger of WDEIA symptoms after ingestion of wheat protein (Yokooji et al. 2013). For the Tri a 21, the α -gliadin allergen, only a few peptide sequences were identified in the digested samples. This protein is responsible for the bakers' allergy (Sander et al., 2015).

Epitopes with a lower frequency matched with Tri a 19 and Tri a 36, show an occurrence that may be ascribed to a high susceptibility to enzymatic digestion. Although, Tri a 36 allergen (LMW-GS) is known to retain IgE reactivity, since it survives the extensive oral, gastric and duodenal in vitro digestion (Baar et al., 2012), a very small number of allergenic epitopes was found in all the considered cvs and N rates, thus suggesting that the amount of Tri a 36 that forms and accumulates in mature seeds was probably quite low for each cv at the time of the collection (Baar et al., 2012). The occurrence of epitope sequences, determined by means of in silico analysis, should be confirmed by means of an appropriate immunological test using the sera of wheat allergic subjects.

3.9. Bioactive peptides

Together with the negative effects on human health, we evaluated any potential positive effects derived from the ingestion of the considered wheat and tritordeum cvs. Numerous digestion derived peptides are known to be precursors of peptides with described antioxidant and opioid effects. The bioactive peptides found in all the digested samples are shown in Fig. 6. The "GYPT" and "YPQPQPF" opioid sequences were identified in all the samples. Gliadorphin 7 (also known as glutomorphin), a δ -exorphin with a YPQPQPF sequence, which is formed during the digestion of α -gliadin, deserves particular attention because its presence could be related to neurodevelopmental disease and psychotic disorders (Liu and Udenigwe, 2019).

"GYYP" was identified in digested flour from the modern cv. For both N rates, and in landrace for the high N application. Peptides containing the "PYPQ" antioxidant sequence were identified in all the samples, while "LQPGQGQG" was only identified in the modern cv.

4. Conclusions

We have investigated the effect of N fertilization on the gluten profile of three genotypes with different gluten compositions. The study has shown that increasing the N rate influenced the GPC to a great extent, while it was found to have less impact on the gluten composition and on the type of celiac and allergenic epitopes after in vitro digestion. On the other hand, these parameters resulted in a greater variation only according to the genotype.

A strong genetic effect was observed with regard to the composition of gluten, especially for glutenins. Tritordeum showed the lowest levels of HMW-GS/LMW-GS and was also distinguished by lower values of ω 5, compared to wheat cvs. Competitive R5 ELISA analysis highlighted that tritordeum may be regarded as a variety with a lower presence of highly CD epitopes than the old and modern wheat varieties that were here considered. The absence of the D genome in tritordeum could be one of the reasons for the lower immunodominant toxicity of this amphiploid.

The study on the expression of celiacogenic and allergenic sequences in different genomes remains of fundamental importance to provide scientific information. This proteomic study paves the way toward a more inclusive study with a larger number of genotypes, grown under the same environmental and agronomic conditions, to better understand their potential immunotoxicity. The qualitative analysis of the epitopes made it possible to observe that in all the studies cv and agronomic conditions (e.g. fertilization rate) the largest number of sequences identified belonging to the class of α - and γ -gliadins, respectively for celiac and allergenic epitopes. The CD epitopes showed a greater presence in the α -gliadin fraction, where a 33-mer is present as strong stimulators of the T-cell response resistant to digestion. All the compared cv and N rate combinations showed quite concordantly a high presence of epitopes belonging to the allergens Tri a 26 and Tri a 21, of HMW-GS and γ -gliadins respectively, and very low for Tri a 19 and Tri a 36, belonging to ω 5 and LMW-gs respectively. These results showed an interesting in-depth proteomic profile of the conditions studied. To verify the quantitative effect of a N rate and its interaction with the genotype, strictly at the epitope level, it would require to build a method to quantify robust information considering the heterogeneity of the genome and the presence of repeated regions in the starting matrix. This will be a future goal of great interest.

The protein profile of tritordeum, compared to the two soft wheat cvs, supports its application as an interesting diet ingredient, given its lower R5 immunogenicity and high GPC content. However, the use of

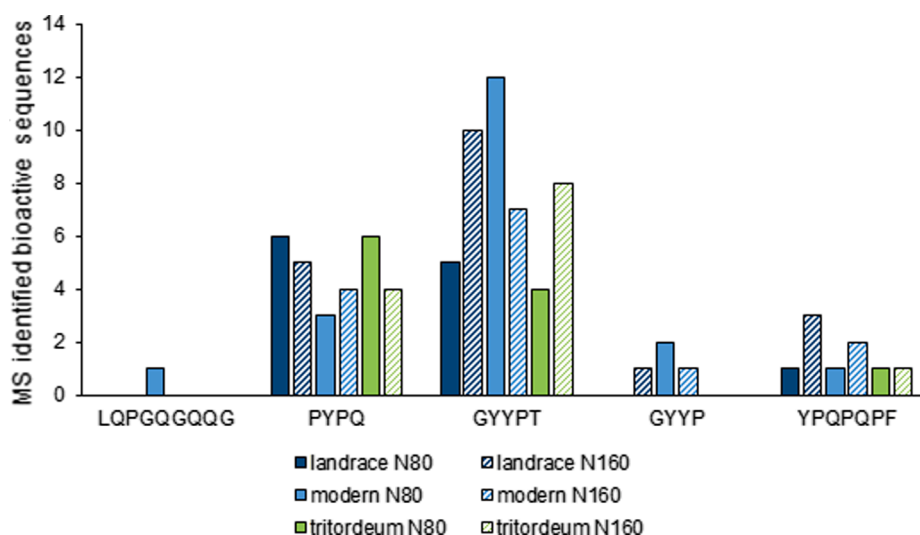


Fig. 6. A graphical qualitative representation of the MS identified epitope sequences inferred to a selection of bioactive peptide in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha⁻¹) and N160 (160 kg N ha⁻¹) fertilization rates.

this new amphiploid species, as a promising alternative to soft wheat, will require specific breeding programs to enhance the end-use quality and immunogenic, nutritional and agronomic traits of such a crop.

CRedit authorship contribution statement

Landolfi Viola: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **D'Auria Giovanni:** Data curation, Formal analysis, Investigation, Methodology. **Nicolai Maria Adalgisa:** Formal analysis, Methodology. **Chiara Nitride:** Writing – review & editing. **Blandino Massimo:** Conceptualization, Funding acquisition, Project administration, Supervision. **Ferranti Pasquale:** Conceptualization, Funding acquisition, Project administration, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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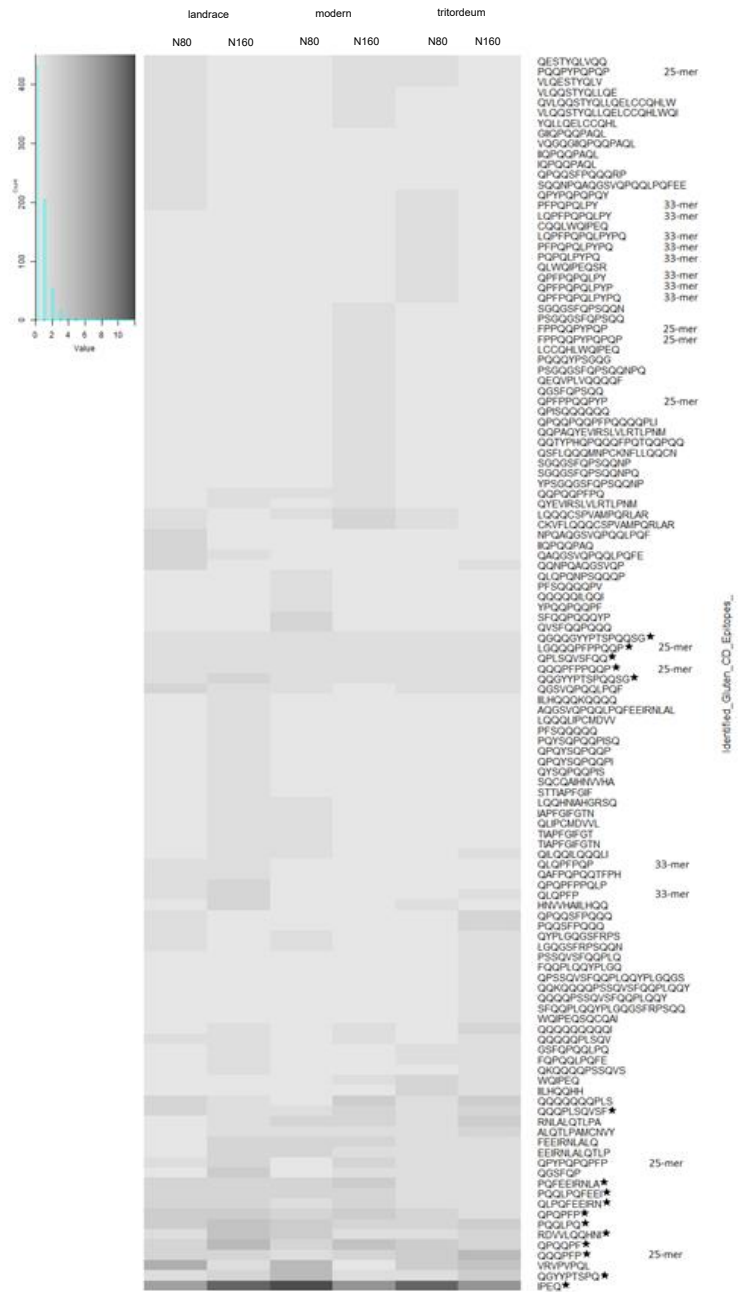
Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2021.110617>.

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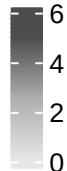


Samples

landrace N80 landrace N160 modern N80 modern N160 tritordeum N80 tritordeum N160



Identified_allergenic_epitopes_intensity



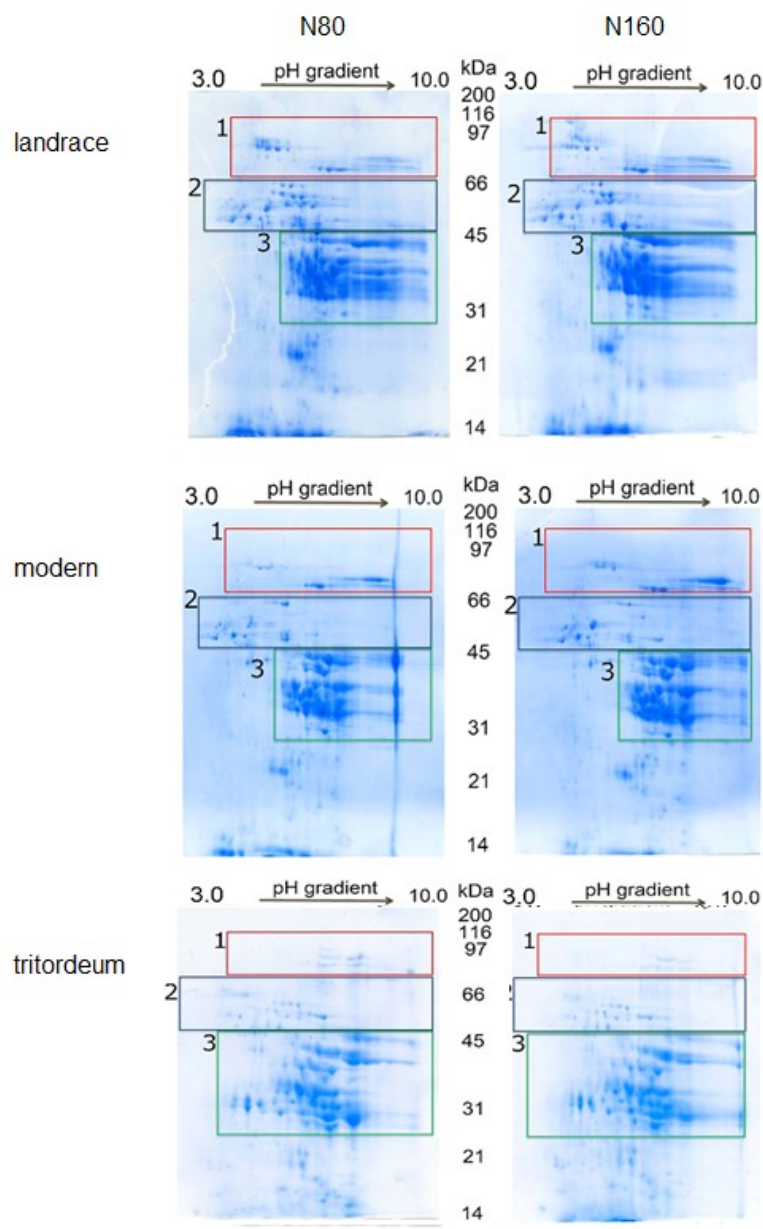
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1Supplementary material

2Supplementary figures

3Figure S1.

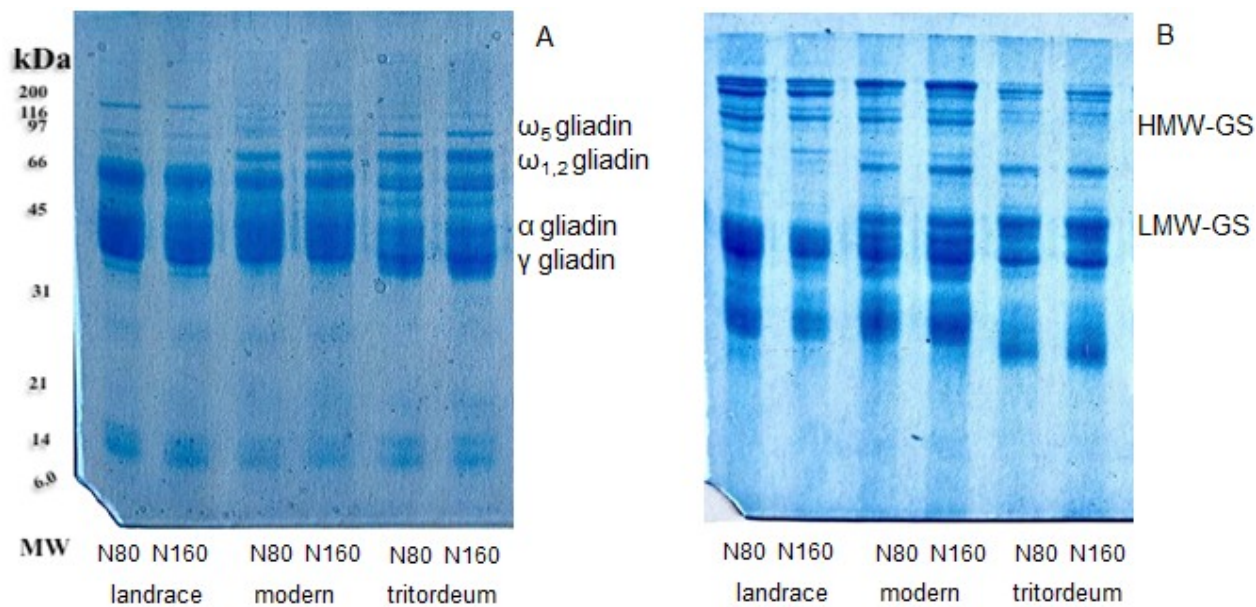
4Two-dimensional electrophoresis analysis of the extracted flour proteins in the soft wheat
5(landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha⁻¹) and N160 (160
6kg N ha⁻¹) fertilization rates.



7
8The protein patterns, as shown in the regions between 20 and 200 kDa, are underlined by the red, blue and
9green colored rectangular areas, in which spots of the same fractions are indicated for HMW-GS, ω5, ω1,2
10gliadins and LMW-GS, α-gliadin and γ-gliadin, respectively.

11**Figure S2.**

12SDS-PAGE analysis of the gliadins (A) and glutenins (B) of the soft wheat (landrace and
13modern) and tritordeum cultivars for the N80 (80 kg N ha⁻¹) and N160 (160 kg N ha⁻¹)
14fertilization rates.

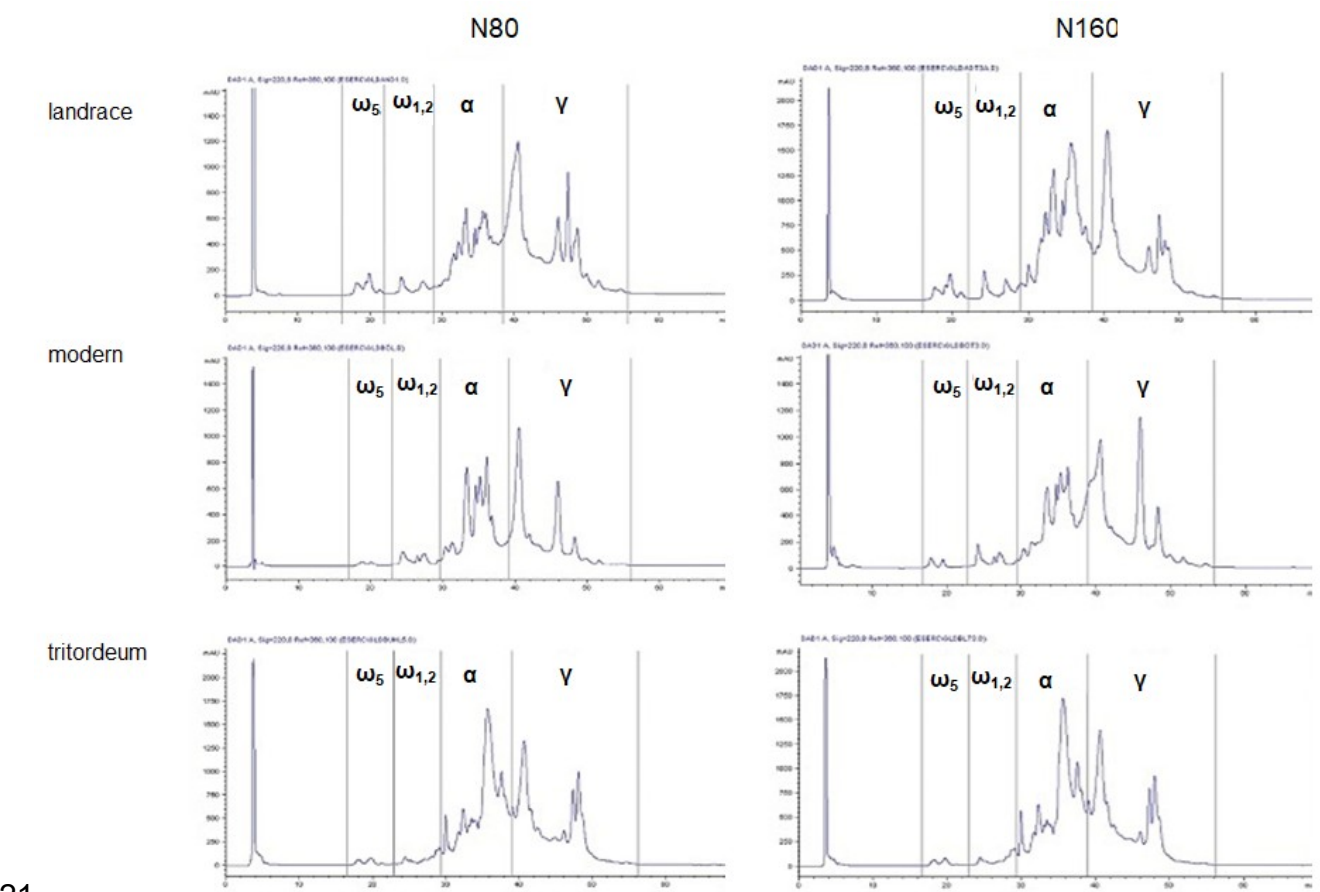


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16

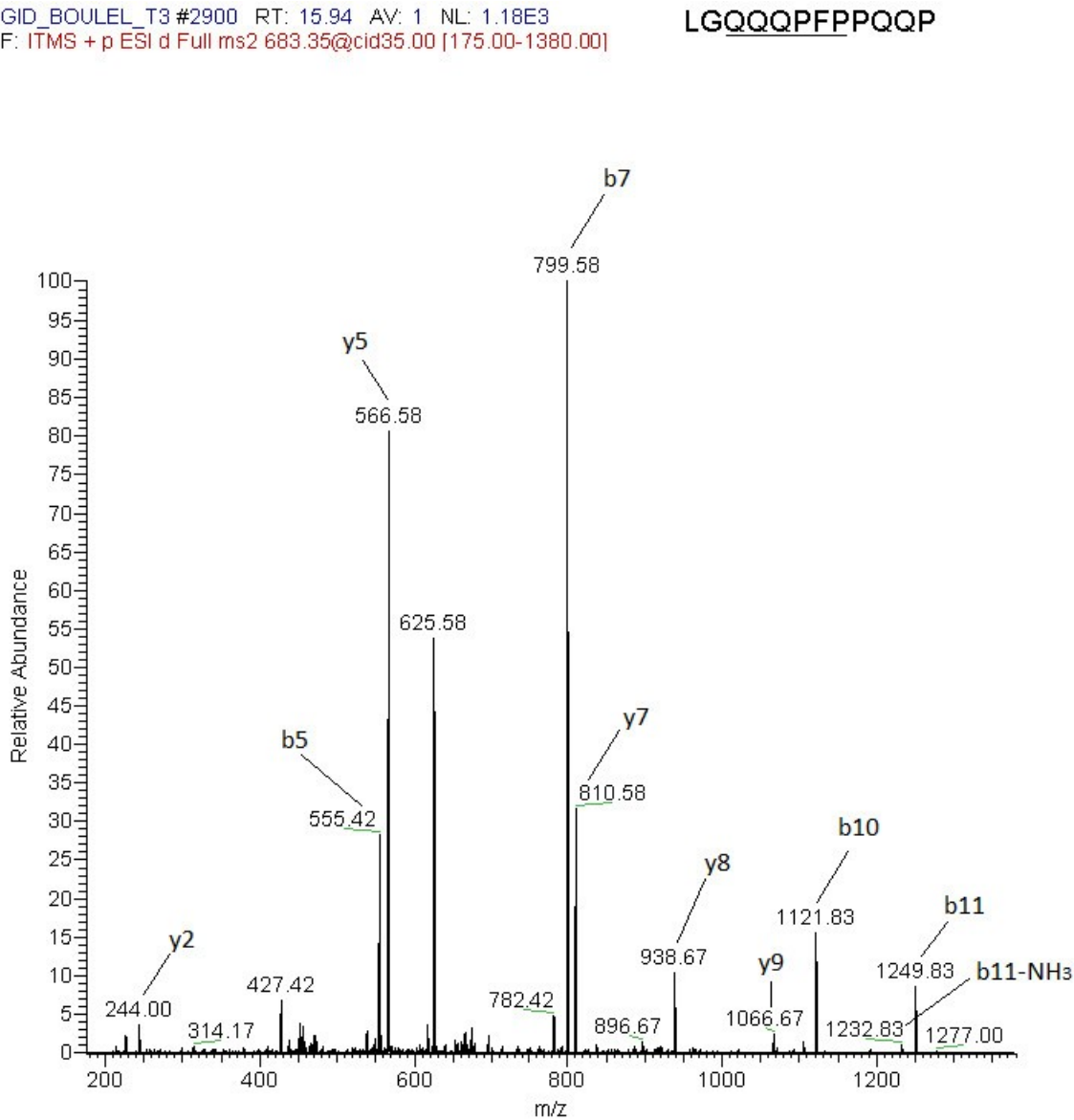
17**Figure S3.**

18HPLC-UV-DAD chromatograms of the gliadins from the soft wheat (landrace and modern)
19and tritordeum cultivars for the N80 (80 kg N ha⁻¹) and N160 (160 kg N ha⁻¹) fertilization
20rates.



22Figure S4.

23Example of a chromatogram of the LGQQQPFPPQQP GID peptide of the tritordeum N160
24sample (fertilization rate of 160 kg N ha⁻¹).



25

26

Figure S5.

Heatmap of the number of CD epitopes in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha⁻¹) and N160 (160 kg N ha⁻¹) fertilization rates.

A matrix that presents the list of epitopes was obtained for each sample divided by gluten protein fractions (the asterisk marks the epitopic sequences present in all the samples).

In attached pdf file

Figure S6.

Heatmap of the number of the allergenic epitopes in the soft wheat (landrace and modern) and tritordeum cultivars for the N80 (80 kg N ha⁻¹) and N160 (160 kg N ha⁻¹) fertilization rates.

This heatmap was built using a matrix that reports the list of epitopes obtained for each sample divided by gluten protein fractions (the asterisk marks the epitopic sequences present in all the samples).

In attached pdf file

42**Supplementary table**

43**Table S1** GID SEQUENCES for tritordeum for N80 in attached excel file

44**Table S2** GID SEQUENCES for tritordeum for N160 in attached excel file

45**Table S3** GID SEQUENCES for modern for N80 in attached excel file

46**Table S4** GID SEQUENCES for modern for N160 in attached excel file

47**Table S5** GID SEQUENCES for landrace for N80 in attached excel file

48**Table S6** GID SEQUENCES for landrace for N160 in attached excel file

49**Table S7**

50Fragmentation spectrum of the 25-mer peptide (LGQQPFQQPYQPQPFPSQQPY) and
5133-mer peptide (LQLQPFQPQLPYQPQLPYQPQLPYQPQPF) sequences with the
52GID celiac peptides of the soft wheat (landrace and modern) and tritordeum cultivars for
53the N80 (80 kg N ha⁻¹) and N160 (160 kg N ha⁻¹) fertilization rates. The letters in red
54represent mismatches in the alignment.

Cultivar	peptide	N rate	
		N80	N160
landrace	25-mer	HQQPFPSQQPYQPQPFPPQ	HQQPFPSQQPYQPQPFPPQ
		LGQQQPFPPQQP	LGQQQPFPPQQP
		RPQQPYQPQPQYSQPQHP	
	33-mer	QLQPFQPQ	QLQPFQPQ
		PFPQPQLPY	QLQPFQ
modern	25-mer	LGQQQPFPPQQP	HQQPFPSQQPYQPQPFPPQ
			LGQQQPFPPQQP
			RPQQPYQPQPQYSQPQHP
	33-mer		GRQQPFPPQQPYQPQPF
tritordeum	25-mer	HQQPFPSQQPYQPQPFPPQ	LQPFQPQLPYQPQH
		LGQQQPFPPQQP	QLQPFQ
		RPQQPYQPQPQYSQPQHP	
	33-mer	LQPFQPQLPYQPQH	LQPFQPQ
		QLQPFQ	QLQPFQ

55

56**Table S8.**

57Example of the alignment of one IEDB allergenic peptide (IEDB epitope sequence:
58QQPGQGQQ in red) with the GID celiac peptides of the soft wheat (landrace and modern)
59and tritordeum cultivars for the N80 (80 kg N ha⁻¹) and N160 (160 kg N ha⁻¹) fertilization
60rates.

Cultivar	N rate	
	N80	N160
landrace	TSRQRSGQGQQPGQGQQSGRGQQG	QGHYPASLQQPGQGQQGHYPT
	TSPRQGSYYPGQASPQQPGQGQQPDK	QQTGQPGQKQQPGQGQQT (A9YSK3)
	QASPQQLGQGQQPGQGQQP	QRQQGQIPASQQPGQGQQGHYP (A9YSK3)
	QGQQGHYPASLQQPGQGQQGHY (GLT0)	GQQGHYPASLQQVGQGQQIGQLGQRQQPGQGQQ
		QGHYLASQQPGQGQQRHYPASLQQPGQGQQGHYTASLQQ (Q0Q5D8)
modern	QGGQGHYPASLQQPGQGQQGHY (GLT0)	QGGQRHYPASLQQPGQGQQGHYTAS (Q0Q5D8)
	QQPGQGQQSGRGQQSGQGH	
	HYPTSLQQLGQGQQIGQPGQKQQPGQGQQT (A4URY8)	QASPQQLGQGQQPGQGQQP
	PTSPQQPGQGQQPGQGQQGHCPMSP (A4URY8)	QRQQPGQGQQP
	QQPGQGQQPRQ	QEQQPGQGQQDQPG (Q18MZ6)
tritordeum	QASPQQLGQGQQPGQGQQP (A5HMG1)	QQPGQEQQPGQGQQDQPG (Q18MZ6)
	SGQGQQGQQPGQGQQAYYPTSSQSGQRQQAG (A5HMG1)	QQPGQGQQPRQ (Q18MZ6)
	QQPGQGQQPGQGQQGHCPMSP	QQPGQGQQPGQGQQGHCPMSPQQTGQAQQGQGQQ
	PGQGQQPGQGQQGHCPMSP (B8PSA6)	QASPQQLGQGQQPGQGQQP
	QRQQPGQGQQP (B8PSA6)	PGQEQQGQQPGQGQQGQQP
	YPASQQPGQGQQGHYPASEQ (B8PSA6)	QQPGQGQQPRQ (A9YSK5)
	GQGQGHYTASLQQPGQGQQGH	QQPGQGQQSGQGQPGYYPTSLR (A9YSK5)

61
62The sequences that have been attributed to proteins with the same accession number, which have only been
63reported to identify sequences belonging to the same peptide, are highlighted in yellow.

64