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Effect of additional water supply during grain filling on protein composition and epitope characteristics of winter oats

Gyöngyvér Gell [a,*,1], Ildikó Karsai [b,1], Zita Berki [b], Ádám Horváth [b], Christakis George Florides [c], Zsófia Birinyi [a,d], Dalma Nagy-Réder [a,d], Balázs Varga [e], András Cseh [b], Ferenc Békés [f], Ottó Veisz [e]

[a] Department of Biological Resources, Agricultural Institute, Centre for Agricultural Research, Eötvös Loránd Research Network, Martonvásár, Hungary
[b] Molecular Breeding Department, Agricultural Institute, Centre for Agricultural Research, Eötvös Loránd Research Network, Martonvásár, Hungary
[c] Murdoch University, College of Science, Health, Engineering and Education, Perth WA, Australia, Perth, WA, 6150, Australia
[d] Doctoral School of Biology, Institute of Biology, ELTE Eötvös Loránd University, Budapest, Hungary
[e] Cereal Breeding Department, Agricultural Institute, Centre for Agricultural Research, Eötvös Loránd Research Network, Martonvásár, Hungary
[f] FBFD PTY Ltd., Sydney, Australia

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ABSTRACT

Pure oats in gluten-free diets (GFD) represent important nutritional benefits for people suffering from celiac disease (CD). However, oat cultivars do not contain the typical CD-related wheat gliadin analog polypeptides. Emerging evidence suggests that oat cultivars containing gluten-like epitopes in avenin sequences may pose potential health risks for celiac patients in rare cases, depending on the individual's susceptibility. Consequently, it is necessary to screen oats in terms of protein and epitope composition, to be able to select safe varieties for gluten-free applications. The overall aim of our study is to investigate the variation of oat protein composition directly related to health-related and techno-functional properties and to examine how the protein compositional parameters change due to irrigation during the grain-filling period as compared to the natural rain-fed grown, in a large winter oat population of different geographic origin.

Elements of an oat sample population representing 164 winter oat varieties from 8 countries and the protein composition of resulting samples have been characterized. Size distribution of the total protein extracts has been analyzed by SE-HPLC, while the 70% ethanol extracted proteins were analyzed by RP-HPLC. Protein extracts are separated into 3 main groups of fractions on the SE-HPLC column; polymeric, avenin, and non-avenin monomeric protein groups, representing 59.17–80.87%, 12.89–31.03%, and 3.40–9.41% of total protein content, respectively. The ratio of polymeric to monomeric proteins varied between 1.71 and 6.07. 91 RP-HPLC-separated peaks have been differentiated from the ethanol extractable proteins of the entire population.

The various parameters identified a lot of variation, confirming the significance of genotypic variation. In addition, it was also established that the additional water supply during grain filling significantly affected the various quantitative parameters of protein content, but not its qualitative structure. This environmental effect, however, was strongly genotype-dependent. Winter oat genotypes with low levels of epitope content were identified and it was proven that these characteristics were independent of the environmental factor of water availability. These genotypes are appropriate for initiating a specific breeding program to yield oat cultivars suitable for CD patients.

1. Introduction

Oat (*Avena sativa*) is unique among all cereal crops because it contains many nutrients that add value to human food and animal feed. Oat varieties are best grown in temperate regions and have lower summer heat requirements and greater tolerance to rain than other cereals (O'Donnell and Adkins, 2001; Hakala et al., 2020).

Consumption of oat products is becoming more and more popular in

* Corresponding author.
E-mail address: gell.gyongyver@atk.hu (G. Gell).
[1] Gyöngyvér Gell and Ildikó Karsai contributed equally to this work.

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the human population and several studies proved that it carries several nutritional benefits including high contents of bioactive compounds such as β-glucans and antioxidants, along with vitamin E and avenanthramides, as well as being an important source of proteins, fats, vitamins, minerals, fibers, phenolic acids, flavonoids, sterols and phytic acid (Zwer, 2010; Pinto-Sánchez et al., 2017; Fritz and Chen, 2018; Jágr et al., 2020). Several clinical studies confirm that the soluble fiber β-glucan is strongly related to lowering blood cholesterol (LDL) levels (Othman et al., 2011; Wolever et al., 2011; Charlton et al., 2012). Besides this, oat consumption can stimulate the immune system and positively affect the function of the human intestinal flora. Since oats are one of the best sources of fatty acids among cereals, especially linoleic acid and low amounts of saturated fat, it plays a significant role in reducing the risk of cardiovascular diseases (Webster, 2011; Van den Broeck et al., 2016).

In recent years, two reviews have been reported indicating the nutritional benefits and features of oats. Shvachko and co-workers highlighted the importance of studying plant genetic resources and the biosynthetic processes of bioactive compounds in oats, which require consistent revision and improvement of methodological and technical approaches (Shvachko et al., 2021). Paudel and co-workers summarized the potential health benefits of oats and their bioactive components regarding various diseases including cardiovascular diseases, type II diabetes, obesity, cancer, immunomodulation, antioxidant activity, gut health, inflammation, atherosclerosis, antimicrobial, dermatological disorder and celiac disease (Paudel et al., 2021).

Apart from preventing different diseases, the increased demand for oat products, makes oats one of the most promising and beneficial crops in the future.

The inclusion of oats in the diet of celiac patients has been a controversial issue. Oats are a less likely candidate to trigger celiac disease due to their protein composition and therefore it has an essential role in the formulation of gluten-free products. On the other hand, all of the important techno-functional properties of oats are directly related to the ratio of polymeric and monomeric proteins in the sample.

CD is an autoimmune disorder triggered by the consumption of gluten proteins of primarily wheat, rye and barley in a part of the population with certain genetic predispositions. The pathological processes induced by gluten in these individuals cause villous atrophy in the small intestines. The prevalence of CD is on average 1% worldwide, making it one of the most common food-related adverse reactions. Currently, the only way to treat CD is to adhere to a lifelong gluten-free diet (GFD) (Scherf et al., 2016; Brouns et al., 2019). By omitting staple cereals, a GFD represents a risk of decreased intake of vitamins (predominantly B group vitamins), important minerals (zinc, magnesium, selenium, and iron) and dietary fiber. The GFD is generally also accompanied by an excess intake of proteins, fats, and sugars.

Wheat prolamins are the key players in the formulation of CD, especially their α- and γ-gliadin subunits (Chand and Mihas, 2006; Catassi and Fasano, 2008). These proteins contain several T cell stimulatory epitopes, mostly in their repetitive regions (Arentz-Hansen et al., 2000; Arentz_Hansen et al., 2002; Shan et al., 2002). In the case of oats, the main storage proteins are the 11S and 12S type globulins, which consist of approximately 80% of the total protein content. The remaining fractions are water-soluble albumins (14–20%) and alcohol-soluble prolamins, named avenins (4–14%), depending on the genotype (Shewry and Halford, 2002).

Oats are in general considered to have low CD-triggering potential due to their lower prolamin content, higher digestibility and lower affinity to MHC (Major Histocompatibility Complex) molecules associated with CD compared to those wheat prolamins (Hoffmanová et al., 2019).

Several clinical studies investigated the safety of oats in GFDs. Despite inconsistent results, more and more studies have shown that the consumption of oats in moderate amounts (20–25 g/day for children and 50–100 g/day for adults) is safe for most celiac patients in remission (Ciclitira et al., 2005; Haboubi et al., 2006; Fric et al., 2011; Rubio-Tapia et al., 2013; Gilissen et al., 2016). A study evaluated the long-term consumption of an oat-based diet by celiac patients and reported no small-bowel mucosal villous damage, inflammation, or gastrointestinal symptoms, with the consumption of an average of 24 g of oat-based diet daily for 8 years (Kaukinen et al., 2013). However, Schmitz and co-workers (Schmitz, 1997) stated that consuming large amounts of oats (100–160 g) daily over a long period might be toxic for patients with CD. A major problem of oat consumption in the celiac context is gluten contamination from other gluten-containing cereals which occurs frequently during conventional agricultural and food processing practices (Thompson, 2004; Lionetti et al., 2018). The problem is being addressed in several countries by developing agricultural and industrial procedures to produce oats free from gluten contamination, referred to as pure oats (Koerner et al., 2011; de Souza et al., 2016; Allred et al., 2017; Smulders et al., 2018). In accordance with the findings described above, the inclusion of pure oats in the GFDs in moderate amounts is recommended by multiple countries, including the EU 828/2014 EU Regulation (828/2014/EU), the U.S. The Federal Regsiter, 2013 and Canada (La Vieille et al., 2016). The legal gluten-free threshold of 20 mg/kg gluten applies to these oat products as well.

Although pure oats are considered to be safe for most celiac patients, there are several studies suggesting that oats may be able to trigger CD on their own, but only affected the minority of the celiac population connected to individual sensitivity and the condition of the intestine (Tuire et al., 2012).

Based on the results of Silano et al. (2014), preclinical and clinical tests with a large number of patients and a control group proved that differences can occur based on certain oat genotypes and individual sensitivity of patients as well. In the tests, increased gliadin-induced transglutaminase enzyme production was observed by fluorescent microscopy on the duodenum segments incubated with protein extracts of wheat and certain oat genotypes. This suggests that not only the contamination of oats with other gluten-containing grains can cause problems, but also there are oat cultivars that contain protein sequences with a low risk for celiac patients. According to the study by Real et al. (2012), there is a great variety of potential immune reactivity of oat cultivars which can generate a higher or lower degree of immune response in patients with celiac disease.

The contradictory preclinical and clinical results and the findings of research aimed at the genetic variability of avenin immunoreactivity (Silano et al., 2014; Comino et al., 2016) suggest that oat varieties are not treated equally in terms of their safety in CD. This has important implications for pure oat production and highlights the importance of screening oat cultivars for the presence of celiac-reactive avenin epitopes. Gilissen and co-workers found that the monoclonal antibody (mAb) G12 developed for gluten detection (Morón et al., 2008; Halbmayr-Jech et al., 2012) cross-reacts with some sequences in avenins, but these peptides were considered irrelevant regarding the presence or absence of the clinically proven toxic internationally agreed celiac epitopes (Gilissen et al., 2016). The researchers suggested it might be a suitable tool for a fast, high-throughput pre-screening of oat varieties (Comino et al., 2011). However, the mAb G12 does not recognize the internationally confirmed oat avenin epitopes (Sollid et al., 2020), but the antibody response is well correlated with the results of T cell proliferation and interferon γ release (Comino et al., 2011). The results of the clinical studies did not support the *in vitro* findings, the reasons possibly being that avenins did not contain any proteolytically resistant peptides longer than 10 amino acids and those avenin peptides have low binding stability on HLA-DQ2.5 (Hardy et al., 2015).

However, to obtain reliable information about the presence of celiac-

related epitopes, immunological results should be accompanied by data on protein composition. The current scientific status about the safety of oats does not provide arguments to categorize certain oat cultivars as potentially harmful regarding CD. LC-MS (Liquid chromatography-mass spectrometry) is the most important tool for the identification and quantification of immunoreactive cereal proteins (Alves et al., 2017). However, the quantification of gluten epitopes with this precise method can still be limited due to the high cereal protein polymorphism and incomplete gluten database of oat immune-responsive proteins (Alves et al., 2019).

The overall aims of our study were (1) to demonstrate the variability of oat protein composition directly related to health-related and techno-functional properties and (2) to examine how the protein compositional parameters change due to irrigation during the grain-filling period, compared to the natural rain-fed grown, in a large winter oat population of different geographic origin. In this report, we summarize our findings related to genetic and environmental factors of the storage protein-related quality that have been analyzed using a complex relatively fast and cost-effective protein separation methodology, suitable for characterizing large sample populations and the resulting data have been evaluated applying published proteomic information. The data collected in this study on the overall protein composition including the ratio of polymeric to monomeric oat proteins can be directly related to functional properties. In addition, the results of the detailed analysis of avenin proteins can help breeders to select oat lines with suitable and relatively stable storage protein composition irrespective of the amount of water available to the plants during grain filling.

2. Materials and methods

2.1. Plant material

In this study, 164 winter oat cultivars and breeding materials of various geographic origins were included in an autumn-sown field experiment. All of the varieties are coded; Supplementary Table 1 contains information on the geographic origins. They have come from eight countries, 79 from the USA, 47 from Hungary, 20 from the UK, 10 from DEU, 5 from POL, and 1-1 from FRA, RUS and UZB. After harvest, the oat seed samples were stored in a dry, chilled warehouse. The grinding of the grains was carried out with a Retsch MM 400 ball mill (Retsch GmbH, Germany) in a gluten-free laboratory environment, which was monitored with the R-Biopharm RIDASCREEN RIDA^{TM}QUICK Gliadin test stripes (Art. No.: R7003).

2.2. Field experiment

The field experiment was carried out in 2017, in the Centre for Agricultural Research, ELKH, Martonvásár, Hungary (Latitude: 47° 21′ N, Longitude: 18° 49′ E, Altitude: 150 m) in an experimental field equipped with an irrigation facility. Two treatments were applied: rain-fed and irrigated, which only differed from each other for the precipitation available during the spring of 2018. Apart from this, the same management practice (optimal fertilization level, pest, weed, and disease control) and the experimental design were used for both treatments. Sowing was carried out on 10 October, in three replicates. Two rows of each genotype were sown in a 0.4 × 2 m plot, and within each plot, the distance between the rows was 20 cm. In a season with average rainfall, five irrigation events were carried out during the grain-filling period using microjets (micro-sprinkler technology). During each irrigation event, additional water corresponding to 10 mm of rain was provided in the irrigated treatment. After full ripening, each plot was harvested and samples for storage protein-related quality examinations were collected from the three replications of both treatments. The details of the weather conditions are shown in Supplementary data 1.

2.3. Protein content

The protein content of oat flours was determined by the Dumas method (N × 5.95) an adaptation of the AOAC Official Method (Association of Official Analytical Chemists, 1995) using an automated protein analyzer (LECO FP-528, USA).

2.4. Protein composition

The effects of irrigation on the protein composition and the amounts of celiac-related epitopes in the samples have been investigated by the analyses of the above-mentioned irrigated (IRR) and rainfed (RF) samples of the 164 genotypes. The relative difference in the amounts of different analytical parameters (x) found in the corresponding IRR and RF samples $\mathbf{[dx = (x_{IR} - x_{RF})/x_{RF}]}$ has been used to monitor the effects and to select genotypes with significant alteration in protein composition and epitope levels.

The analytical procedures applied in this study were identical to those used and described in depth by our previous publication (Gell et al., 2021), using the same methods, equipment, and separation procedures used by the same operator.

A simplified version of the procedure of Gupta et al. (1993) was applied as a one-step extraction followed by the SE-HPLC separation method of Larroque and Békés (2000) to determine the amounts of avenin content of the samples as well as amounts of polymeric and monomeric non-avenin proteins expressed as the percentage of the total protein content of the samples.

The RP-HPLC procedure of Larroque et al. (2000) has been applied to characterize the avenin composition. 60 mg oat flour was extracted using 70% ethanol and vortexed in a horizontal vortex (Vortex-Genie^{TM} 2, MO BIO Laboratories, Inc. USA) at setting 6 for 30 min. Samples were centrifuged for 15 min at 13 000 rpm g using Eppendorf Centrifuge 5424. The supernatant was aspirated with taking care of the pellet and passed through a 0.45 μl filter into an HPLC glass vial. The samples were prepared in triplicate and were centrifuged for 20 min at 15870×*g*. The supernatant was filtered using a 0.45 μm filter. The protein extracts were separated using an Agilent 1200 LC Systems (Agilent Technologies, USA) by the method of Larroque et al. (2000). 10 μl of extracts were injected into a C18 reversed-phase ZORBAX 300SB-C18 column (4.6 × 150 mm, 5 μm, 300 Å, Agilent Technologies, USA) maintained at 60 °C column temperature and at 50 bar column pressure. The applied eluents were 67% ultrapure water (Buffer A1) and 33% acetonitrile (Buffer B1), each containing 0.1% TFA (HPLC grade, Sigma Aldrich). The separation was carried out using a linear gradient from 33 to 80% Buffer B1 over 65 min at a flow rate of 1 ml/min.

RP-HPLC analyses have been carried out with three replicate injections from two replicate extracts.

2.5. Prediction of avenin-epitope levels

The analytical data derived from the RP-HPLC-based separation provides the basis to predict the individual and cumulative amounts of DQ2.5-ave-1a (PYPEQEEPF), DQ2.5-ave-1b (PYPEQEQPF) (Vader et al., 2003; Arentz-Hansen et al., 2004), DQ2.5-ave-1c (PYPEQEQPI) (Hardy et al., 2015), and DQ2.5-ave-2 (PYPEQQPF) epitopes of oat using the procedure of Gell et al. (2021) based on the detailed proteomic data of Tanner et al. (2019).

Briefly, Tanner et al. carried out RP-HPLC analysis from an Australian oat variety (cv. Wandering). The representative RP- HPLC chromatogram of the purified oat protein sample contained 18 well-defined RP peaks. RP-HPLC fractions were collected from the purified avenin

sample and using MALDI-TOF-MS, and LC-MS/MS analysis of the chymotrypsin-digested samples was carried out for protein identification. RP-HPLC analysis in this study has been carried out using the identical protocol in the same laboratory by the same operators as reported by Tanner et al. (2019) resulting in matched elution profiles of avenin peaks with the published data and those derived from this study. The mass spectrometric information on the avenin peaks eluted at certain retention times of Tanner's work has been adopted to characterize the corresponding RP-HPLC peaks in our study.

The individual and cumulative amounts of avenin proteins containing the four oat avenin T cell epitopes have been determined by selecting and summing the peak intensities based on the retention times of the peaks, expressed in [mg/100 g avenin] units using the average molecular mass of avenin proteins as 29 kDa (Comino et al., 2016) and with the molecular mass values of the four avenin epitopes, calculated from their amino acid composition and finally converted to [mg/100 g sample] units by multiplying the mg/100 g avenin values by the SE-HPLC based avenin content and by the protein content of the samples.

Despite the valid and serious limitations of the prediction method discussed in depth by Gell et al. (2021) - with the lack of any other (better) relatively high throughput and cheap method that applies to large sample populations – the method is suitable to be used as a pre-selection screening tool in oat breeding.

2.6. Statistical analyses

The basic statistical characterization (mean values, standard deviation and coefficient of variation) of three replicate data derived from the three injections of protein extracts in the cases of both SE- and RP-HPLC analyses. The calculations have been carried out using MS Excel functions. Sample groups have been characterized by the variation of the above-mentioned mean values of different protein composition data. To avoid any possible confusion, different notations are used for describing the variation among the replicate measurements of a given sample, (**mean**, **stdev** and **cv**) and the variation among the means of different measurements in a group of samples (**Mean**, **StDev** and **CV**)

In the case of parameters derived from more than one, standard deviations were calculated based on the Gaussian error propagation law (Skoog et al., 2007) from the means and standard deviation values (**σ**) from the individual parameters: in the case of the cumulative amount of epitopes, the geometrical mean of the four standard deviations was used while the following equation was used for the determination of the standard deviation of the avenin levels in mg/100 g sample units:

$$\sigma_{\text{amg/100 g sample}} = 10^{-4} * \mathbf{mean}_{\text{protein}} * [(\sigma_{\text{avenin}})^2 * (\mathbf{mean}_{\text{cum.epitop}}) + (\sigma_{\text{cum.epitop}})^2 * (\mathbf{mean}_{\text{avenin}})^2]^{0.5}$$

While the mean values of the $\mathbf{d_x}$ relative differences of different chemical parameters.

(**mean** $\mathbf{d_x}$) can be calculated directly from the replicate measurements of IRR and RF samples - $\mathbf{mean\ d_x = (mean\ x_{IRR} - mean\ x_{RF})/mean\ x_{RF}}$, the estimation of the standard deviation of this parameter is not trivial.

If $\mathbf{x_{IRR1}}$, $\mathbf{x_{IRR2}}$ and $\mathbf{x_{IRR3}}$, the three replicate measurements for parameters x of the IRR samples, $\mathbf{x_{RF1}}$, $\mathbf{x_{RF2}}$ **and** $\mathbf{x_{RF3}}$, are the three replicates for parameters x of the RF samples, and if $\mathbf{x_{IRR1} > x_{IRR2} > x_{IRR3}}$, and $\mathbf{x_{RF1} > x_{RF2} > x_{RF3}}$, the interval in which the value relative difference can vary is $\mathbf{(x_{IRR3}\text{-}x_{RF1})/x_{RF1} > d_x > (x_{IRR1}\text{-}x_{RF3})/x_{RF3}}$. Fictive arbitrary $\mathbf{d_x}$ values can be generated in this interval to satisfy the real **mean** $\mathbf{d_x}$ values, and using them as "replicate" $\mathbf{d_x}$ values, in statistical analyses as ANOVA or determining the LSD value to compare $\mathbf{d_x}$ values for different genotypes but these results can only be assumed as the extreme lowest possible F values for ANOVA or the extreme highest upper limit values for LSD.

A much more exact way to estimate the variation of $\mathbf{d_x}$ relative differences is by applying the Gaussian error propagation law (Skoog et al., 2007), calculating **stdev(**$\mathbf{d_x}$**)** using the **stdev(**$\mathbf{x_{IRR}}$**)** and **stdev(**$\mathbf{x_{IRF}}$**)**, derived from the replicate measurements on $\mathbf{x_{IRR}}$ and $\mathbf{x_{RF}}$ data with the following formulae:

$$\mathbf{stdev^2(d_x) = (r^2/k^2)*(1/n_{IRR}+1/n_{RF})},\ \text{where}$$

$$\mathbf{r^2 = [(stdev^2(x_{IRR})*(n_{IRR}\text{-}1)+(stdev^2(x_{RF})*(n_{RF}\text{-}1)]/(n_{IRR}+n_{RF}\text{-}2)},\ \mathbf{k^2 = [mean(x_{IRR})*n_{IRR}+mean(x_{RF})_{mean}*n_{RF}]/(n_{IRR}+n_{RF})}$$

where $\mathbf{n_{IRR}}$ and $\mathbf{n_{RF}}$ are the number of replicate measurements on the IRR and RF samples, respectively.

This second approach does not provide "replicate" $\mathbf{d_x}$ values, so ANOVA or similar statistical tests cannot be used, however – using the calculated **mean(**$\mathbf{d_x}$**)** and **stdev(**$\mathbf{d_x}$**)** values – the relative differences found for different genotypes can be compared by statistical *t*-test as well as genotypes with significant alteration of parameter x caused by irrigation can be determined: the criteria of significant alteration is $\mathbf{abs\ [mean(d_x) > 3*\ stdev(d_x)}$

To distinguish the possible patterns in the protein compositional parameters of the oat genotypes, higher order statistical analyses were carried out with the STATISTICA software package, version 13.5.0.17 (TIBCO Software Inc.). Principal Component Analysis was applied to the data matrix of 164 genotypes x 10 phenotypic traits including both the rain-fed and irrigated average values of protein, polymer, monomer, avenin and epitope contents. K-means clustering protocol was applied for establishing the groupings of the genotypes. The option of maximizing the initial between-cluster distances was set, and the most likely cluster number was evaluated in the range of 2–10 clusters, with 10 iterations for each round. The most likely cluster number was determined based on the changes in the sum of the within-cluster distances from the cluster means at each cluster number increase. The correctness of the established cluster number was checked on the same data set by applying the forward stepwise module of discriminant analysis in STATISTICA software using the cluster positions of the cultivars as a dependent variable and the five protein parameters as independent variables.

3. Results

3.1. Effects of irrigation on the protein composition

The effect of additional water on protein composition has been investigated by a two-level characterization of proteins in the oat samples grown under irrigated (IRR) and not irrigated, rainfed (RF) conditions. The distribution of the total protein content after size-based separation was determined with SE-HPLC, resulting in the amounts of avenins and polymeric and monomeric non-avenin proteins expressed as a percentage of total protein content. RP-HPLC based separation was used to characterize the qualitative and quantitative composition of the avenin proteins isolated by 70% ethanol extraction of the samples.

3.1.1. SE-HPLC analysis

As it was realized in our recent previous study on oat proteins (Gell et al., 2021), more than 99% of the total amount of oat flour proteins can be extracted in the first step of the extraction procedure of Gupta et al. (1993), without applying sonication. Comparison of samples has been carried out, therefore, using this simplified, one-step procedure.

The qualitative comparison of SE-HPLC profiles of IRR and RF samples did not show any differences (Supplementary Fig. 1A): altogether 10 peaks, from which two major peaks - P2 and P6 with retention

times: 6.4 and 9.6 min– and some minor ones have been observed on each chromatogram. Following the procedure used by Gell et al. (2021), the amounts of the three main protein groups have been determined by using the analytical data based on the SE-HPLC separation. The polymeric protein fraction consisted of five well-defined peaks (P1–P5) with retention times of 5.2, 6.4, 7.4, 7.9, and 8.3 min, respectively. The next main group is the avenin-type proteins, eluted as P6 (retention time: 9.6 min), while the third group, contains a rather complex mix of the monomer globulin proteins (P7–P10), eluted in the region of 10.0–12.0 min.

The reproducibility of the peak intensity measurements has been monitored by calculating the mean, stdev and cv values for each peak from their 3 replicate analysis data. The averages of cv values calculated for the 164 IRR and RF samples did not show relevant differences. The overall errors for the polymeric, avenin and non-avenin monomeric protein group measurements for both sample groups are 5.28%, 5.73% and 7.44%, respectively.

The distribution of the proteins among the three main groups, using the means of the 3 replicate analyses (Supplementary Table 1), shows a well-defined trend all around the 164 cultivars in both the IRR and RF samples. Using the data of basic statistical comparisons (shown in Table 1), the polymeric fraction represents about three-quarters of the total protein content, for the IRR samples.

One-way ANOVA shows significant inter-cultivar variation for each protein parameter among samples in both IRR and RF treated samples (Table 1). F values found for the different parameters of both IRR and RF samples indicate that the variation in the avenin- and monomer protein ratio are much higher than in the amount of the polymeric fraction and protein contents.

As expected and shown by the linear correlation coefficients between parameters of the same cultivar in the IRR and RF group, there are reasonably strong inter-relationships between the protein parameter values determined in the two treatment groups (Fig. 1A, 2A, 3A 4A, 5A; $\mathbf{r_{protein}} = 0.921$, while r values for the different SE-HPLC separated groups are around 0.5, each of them significant (Table 1).

The similarity between the mean values found for the two treatment groups could lead to the false conclusion that irrigation has only marginal effects on the protein content and composition of oats. In reality, it is more complex, and this can be realized if the compositional data are compared individually from cultivar to cultivar. As it is demonstrated in Figs. 1A–5A, protein content and protein compositional parameters of samples altered positively in some cultivars (for example for protein content: $prot_{IRR} > prot_{RF}$), while the data of other cultivars show the opposite effect ($prot_{IRR} < prot_{RF}$). If the distance of a data point from the 45° diagonal on these figures is larger than the experimental error found for the given measurement, cultivar-dependent positive or negative alteration occurs caused by irrigation. These positive and negative effects are neutralized by calculating the mean values shown in Table 1.

A significant effect of irrigation was found for each parameter based on the two-way ANOVA analyses (Table 2), even the cultivar x treatment effects are highly significant. Comparing the resulting F values, $\mathbf{F_{cultivar}}$ values are a minimum of three times larger than those for the treatment and four times than those for the cultivar × treatment interaction for each protein parameter, $\mathbf{F_{monomer} > F_{avenin} > F_{p/m\ ratio} > > F_{polymer} > F_{protein}}$.

To monitor these cultivar-dependent effects of irrigation, the relative differences of protein parameters (X) found in the irrigated and rainfed samples $\mathbf{d_X = (X_{IRR} - X_{RF})/X_{RF}}$ have been introduced. The $\mathbf{d_x}$ parameters are valid indicators of the alteration caused by irrigation and their values are not interrupted by the reasonably large inter-variety differences of the X parameter in the untreated (RF) samples.

The mean values of $\mathbf{d_x}$ relative differences for each cultivar can easily be determined from the replicate analyses of the IRR and RF samples for the X parameter. These values are shown in Figs. 1B–5B, respectively. Because $\mathbf{d_x}$ is not a direct measurement, it is calculated from the two independent measurements on irrigated and rainfed samples, its standard deviation ($\mathbf{stdev_x}$) is the function of the variation of $\mathbf{x_{IRR}}$ and $\mathbf{x_{RF}}$ replicate measurements and can be determined based on the Gaussian error propagation law.

The three different colors of the bars on color codes (orange, blue, black) in Figs. 1B–5B indicate positive significant, negative significant and not significant differences of the given values from zero, respectively, based on the $\mathbf{abs[mean(d_x)] > 3^* stdev(d_x)}$ criteria. A critical value of $\mathbf{abs[mean(d_x)]}$ was found for each protein parameter above and under which the effect of irrigation is significant (**Table 3**). However, in certain cases – indicated by red arrows in Fig. 3B - when $\mathbf{stdev(d_x)}$ for a given sample was lower than average, a significant difference from zero can be observed at lower than the critical $\mathbf{abs[mean(d_x)]}$ values.

3.1.2. RP-HPLC analysis

A characteristic RP-HPLC pattern is shown in Supplementary Fig. 1B. Peak distribution showed great inter-genotype variation in the number and composition of different avenin polypeptides indicating the extent of genetic and proteomic diversity in this large oat population (Supplementary Table 3). Irrigation altered only the relative amount of polypeptides in the case of most of the genotypes but never changed altered their number or elution times.

In the 164 oat samples, the PATMACH software has matched 91 distinct peaks in the 25.50–54.07 min elution time interval using a 0.10 min window to identify the corresponding peaks in the different chromatograms. The 0.10 min cut-off means that if the difference between the observed retention times of a single peak was lower than 0.10 min then the two peaks have been evaluated as identical peaks. Using this procedure, the number of peaks in a given sample has been determined indicating a large variation between 7 and 21 peaks (Mean 12). This

Table 1
Basic statistics (mean, standard deviation, minimal and maximum values calculated from three replicate analyses, as well as one-way ANOVA on cultivars) investigating the effects of irrigation on the protein content and composition as well as epitope content of the 164 oat samples.

								One-way ANOVA on cultivars	
			mean	stdev	min	max	r	F	p
PROTEIN	g/100 g sample	RAINFED	13.34	1.14	10.93	17.20	0.921	3.27	0.00
		IRRIG	13.41	1.18	10.80	17.65		2.98	0.00
POLYMER		RAINFED	72.06	4.47	61.70	80.87	0.484	5.24	0.00
		IRRIG	71.74	4.07	61.18	79.20		3.96	0.00
AVENIN		RAINFED	21.46	3.96	13.99	31.03	0.501	38.08	0.00
		IRRIG	21.88	3.83	12.89	34.61		37.33	0.00
MONOMER		RAINFED	6.48	1.25	3.40	9.41	0.465	39.50	0.00
		IRRIG	6.44	1.20	3.92	9.38		36.70	0.00
POLYMER to MONOMER ratio		RAINFED	3.51	0.83	2.00	5.70	0.480	28.27	0.00
		IRRIG	3.41	0.78	1.97	6.07		26.41	0.00
EPITOPE content	mg/100 g sample	RAINFED	62.31	31.57	8.53	157.36	0.886	1026.60	0.00
		IRRIG	64.51	32.16	13.44	169.31		986.40	0.00

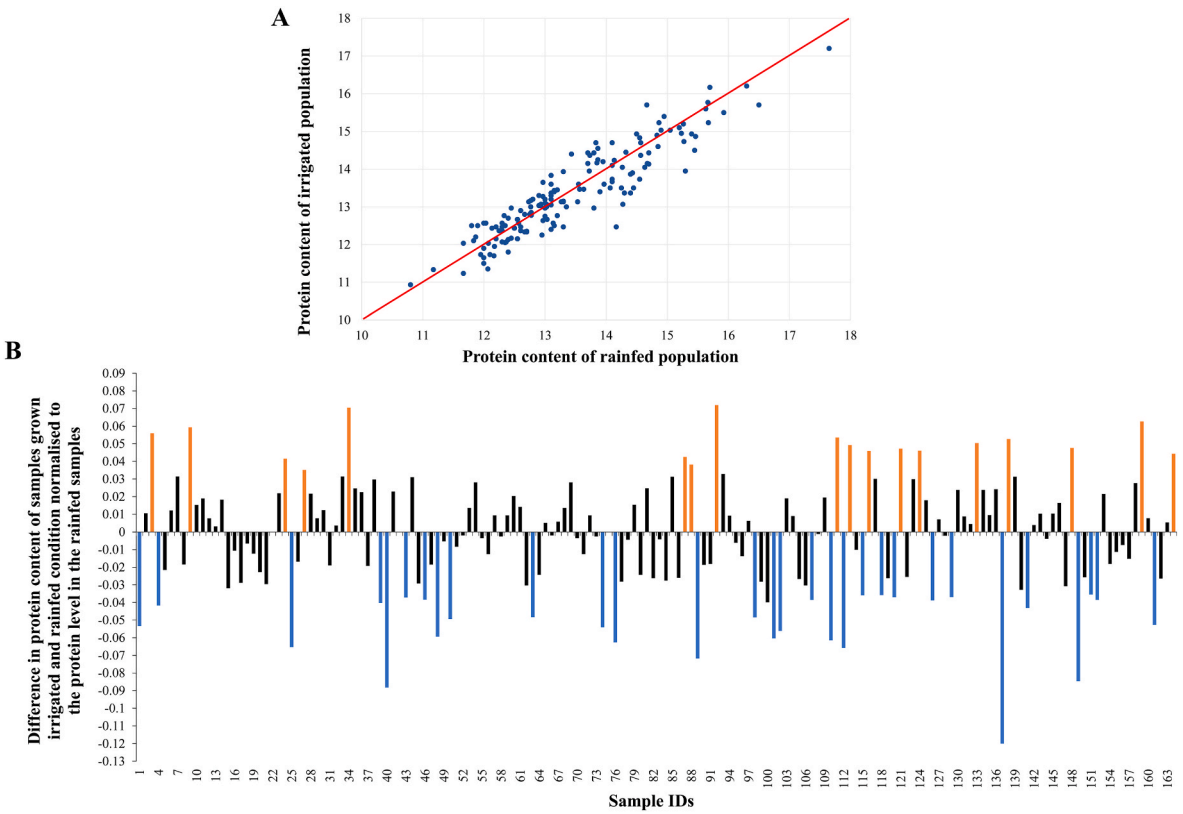

Fig. 1. The effect of additional water supply on the protein content of samples (% of the sample) grown under rainfed and irrigated conditions. A) Comparison of protein contents of samples. Data points above or under the 45° diagonal drawn in red indicate alteration caused by irrigation. (B) The difference in protein content between samples grown under rainfed and irrigated conditions was normalized to the rainfed data [$\mathbf{d}_{\mathbf{protein}}$ = (IRR-RF)/RF]. Bars drawn in black represent no significant alteration; while orange and blue bars show significant positive or negative changes in protein content where the absolute value of means of **d** relative difference calculated from the replicate measurements is three times larger than its standard deviation. Detailed values are shown in Supplementary Table 2. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

variation in the number of separated peaks can be explained by the variability of the resolution of the RP-HPLC technique as the function of the amounts of proteins in a peak. The individual peaks in certain cases might contain more than one protein type (as it was shown in the work of Tanner et al. (2019), characterizing individual RP-HPLC peaks by using the mass spectrometric methodology.

As it was observed in previous studies (Tanner et al., 2019), most of the avenin polypeptides are eluted in two elution time intervals: 20 peaks have been found in the 25.5–32 min interval and 37 in the 38–54.0 min interval, representing the 45.58 and 48.42% of the total avenin content, respectively.

The number of appearances of a peak with a given retention time in different samples was found to be extremely variable. There are rare peak types, only a few genotypes carry, as 7 peaks found only in three cultivars,17 peaks have been identified which appeared in less than 6 samples, while the peak with the retention of 41.65 min was found in 89 samples, seems commonly and uniformly occurring in different oat varieties.

The reproducibility of the peak intensity measurements has been monitored through the 1956 peaks found in the whole sample population by calculating the mean, stdev, and cv values for each peak from their replicate analyses data, resulting in a 7.41% for average value for the cv values. The reproducibility of peak intensity did not show any relationship with the elution time, (the r^2 value between elution time and cv values of peaks eluted at a given elution time was found to be 0.0042), while a strong negative correlation was found between the peak intensities and their reproducibility ($r^2 = 0.8434$): in the 10–15% peak intensity interval, the cv values are smaller than 6%, while in 6–7% and 10–11% in the 5–10% and the >15% intensity intervals, respectively. These values are almost identical to those reported in our previous study (Gell et al., 2021).

The quantitative composition of the avenin proteins shows wide variation among the relative amounts of the different polypeptides: relative amounts of polypeptides with the same elution times varied between 0.1 and 26% (Supplementary Table 3).

A comparison of the composition of avenin samples indicated an identical number of avenin peaks in IRR and RF samples with sometimes large intensity differences. These alterations of protein expression caused by irrigation do not show a uniform pattern: proteins eluted at a given time could show a significant increase in the irrigated sample and a significant drop in another sample. A weak trend can be observed, however: the mean values of the relative difference between the amounts of protein peaks in the IRR and RF samples eluted at a given time, $(\mathbf{c}_{\mathbf{IRR}}\text{-}\mathbf{c}_{\mathbf{RF}})/\mathbf{c}_{\mathbf{RF}}$, show a relationship with the elution time, so irrigation has a larger effect on the relative amounts of less hydrophobic avenin proteins ($r^2 = 0.453$) (Fig. 6).

3.2. *Predicting the amount of celiac-related oat epitope containing components*

Using the data set presented by Tanner et al. (2019) for the composition of avenin fraction of the oat variety cv. Wandering, the amounts of the celiac-related oat epitope containing components of the 164 oat samples have been predicted based on their RP-HPLC analysis results.

Several dominant peaks were identified containing conserved avenin types: peak 8 (R.T. = 28.209 min) in 30 samples, peak 17 (R.T. = 30.437min) in 39 samples, peak 20 (R.T. = 31.053 min) in 33 samples,

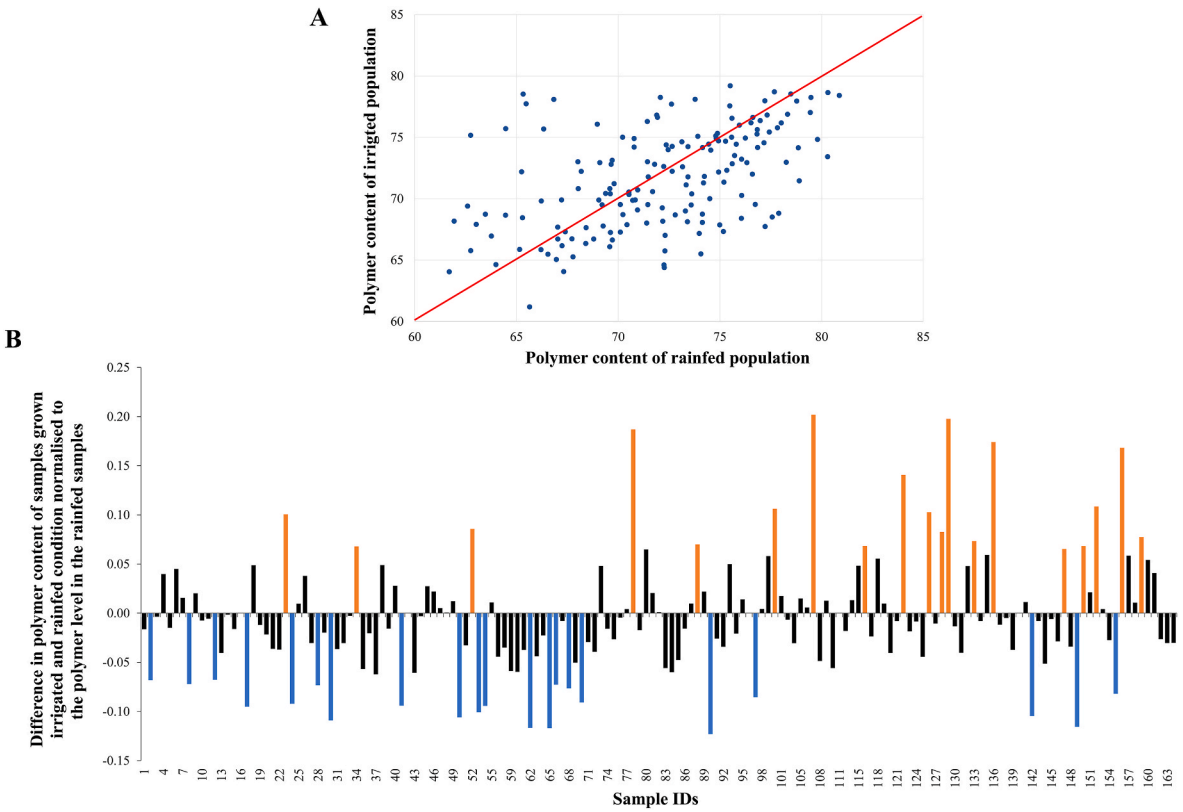

Fig. 2. The effect of additional water supply on the polymer protein content of samples (% of total protein) grown under rainfed and irrigated conditions. A) Comparison of polymer contents of samples. Data points above or under the 45-degree diagonal drawn in red indicate alteration caused by irrigation. (B) The difference in polymer protein content between samples grown under rainfed and irrigated conditions was normalized to the rainfed data [$\mathbf{d_{polymer}}$ = (IRR-RF)/RF]. Bars drawn in black represent no significant alteration; while orange and blue bars show significant positive or negative changes in polymer content where the absolute value of means of **d** relative difference calculated from the replicate measurements is three times larger than its standard deviation. Detailed values are shown in Supplementary Table 2. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

peak 66 (R.T. = 44.133 min) in 8 samples, peak 67 (R.T. = 44.361 min) in 31 samples, peak 72 (R.T. = 45.615 min) in 47 samples, and peak 56 (R.T. = 41.651 min) in 89 samples. According to the nanoLC-MS/MS-based protein identification of the eluates separated by RP appropriate retention times defined by Tanner and coworker (2019), peak 8 contained the gliadin-like avenin (L0L6J0), peak 17 contained also gliadin-like avenin (L0L6K1), peak 20 contained Asat-Prolamin10 protein and a 23539 Da avenin (Q09072), peak 67 contained an avenin-F protein with an alternative name Celiac immunoreactive protein 2 or gamma-avenin-3 (Q09097) and an Asat-Prolamin71 protein, the peak 16 contained avenin (I4EP54), gliadin-like avenin (L0L6J0) and an Asat-Prolamin15 protein. In the case of peaks 8, 17, and 20 the predominant avenin epitope is the DQ2.5-ave-1a (PYPEQEEPF), in peak 67 the DQ2.5-ave-1b (PYPEQEQPF) and DQ2.5-ave-1c (PYPEQEQPI) while in peaks 67 and 72 all the above mentioned three avenin epitopes occurred.

The individual and cumulated amounts of avenin epitopes - determined by selecting and summing the RP-HPLC data according to their retention time, then converting the resulting values to epitope contents based on their molecular mass – are tabulated in Supplementary Table 4.

Large inter-cultivar variation in the epitope content of samples grown both in IRR and RF conditions has been observed (last lines of Tables 1 and 2: mean values of cumulative epitope content varied between 8.53 and 157 mg/100 g sample in the RF samples, and between 13.44 and 169.31mg/100 g samples in the IRR. Based on the correlation coefficients, the relationship between the corresponding values in the two populations (r = 0.886) is significantly stronger than found for the protein parameters. (Fig. 7A).

Comparing the mean values of the different samples (RF = 62.31, IRR = 64.51), a slight increase in the epitope content can be observed. However, comparing the data of the individual cultivars, the alteration caused by the irrigation is more severe: the $\mathbf{d_X = (X_{IRR} - X_{RF})/X_{RF}}$ values vary between −0.5 and 0.6. In 16 cultivars, a significant drop in epitope content can be detected, while the IRR samples contain a significantly larger amount of epitopes than the RF samples in 29 cultivars. (Fig. 7B, Table 3).

3.3. Grouping the winter oat genotypes by their storage protein-related quality parameters

Since the effect of genotype on all protein traits was highly significant, the phenotypic data matrix of the 164 oat cultivars was subjected to further analyses in order to be able to identify distinct groups of oats with specific quality attributes related to storage protein composition. In the K-mean clustering protocol, the probability of five separate groups was the highest, and the correctness of the grouping was supported by the General Discriminant Analysis at a high significance level ($P < 0.00001$); of the 164 oats, the group position of only 3 genotypes remained unresolved. To display the distribution of each quality group, we performed a principal component analysis on the phenotypic data matrix, where the genotypes were labeled with the cluster number they belong to (Fig. 8). Factor 1 of the PCA plot with an Eigenvalue of 3.81 explained 38.15% of the phenotypic distribution.

It showed a strong positive correlation with the polymer content (0.86 and 0.78 in RF and IRR, respectively), and strong negative correlations in decreasing order with avenin (−0.79 and −0.74 in Rf and IR, respectively), epitope (−0.66 and −0.66) and monomer (−0.57, and −0.32) contents. Factor 2 with an Eigenvalue of 2.5 explained 25.0% of the phenotypic distribution and it showed the highest positive

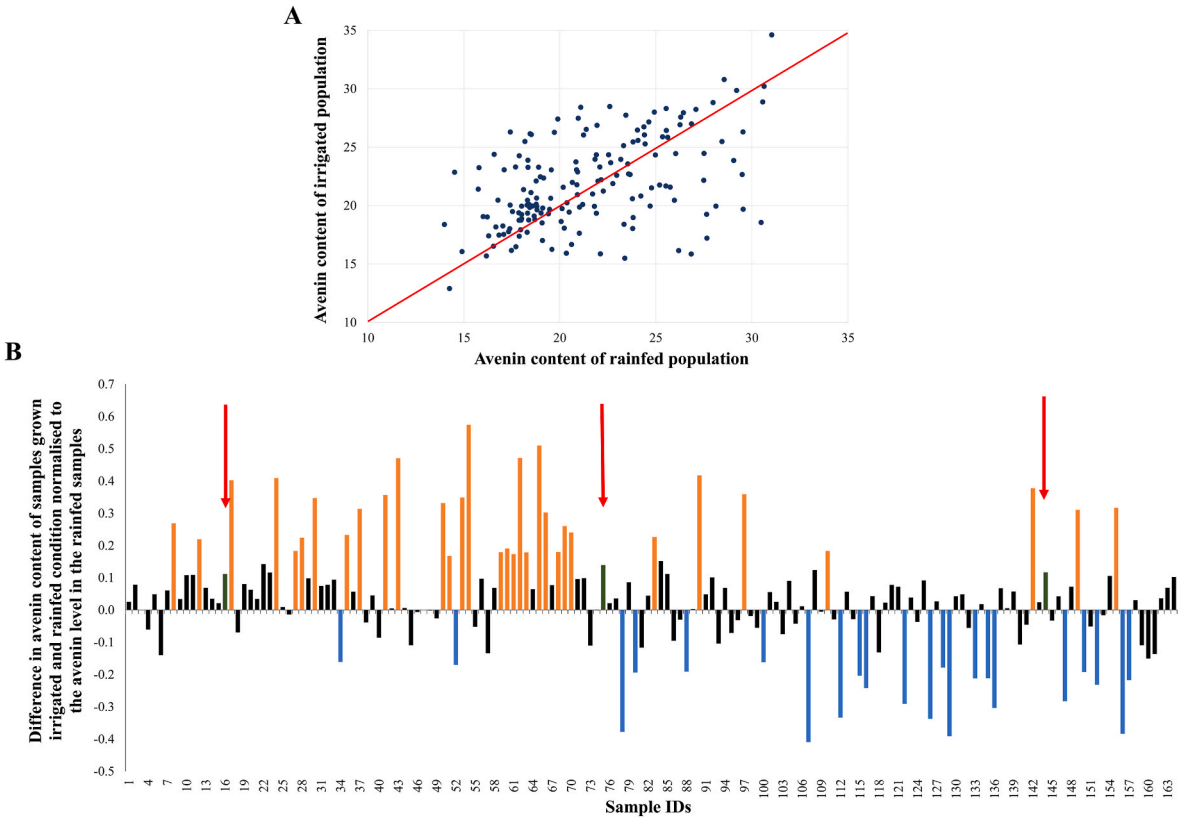

Fig. 3. The effect of irrigation on the avenin content of samples (% of total protein) grown under rainfed and irrigated conditions. A) Comparison of avenin contents of samples. Data points above or under the 45-degree diagonal drawn in red indicate alteration caused by irrigation. (B) The difference in avenin content between samples grown under rainfed and irrigated conditions was normalized to the rainfed data [$\mathbf{d_{avenin}}$ = (IRR-RF)/RF]. Bars drawn in black represent no significant alteration; while orange and blue bars show significant positive or negative changes in avenin content where the absolute value of means of **d** relative difference calculated from the replicate measurements is three times larger than its standard deviation. Red arrows indicate samples with lower than the average standard deviation of d values. Detailed values are shown in Supplementary Table 2. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

correlation with the protein content, irrespective of the treatments (0.89 vs. 0.90). Factor 2 has also a moderate positive correlation with the epitope content in both treatments (0.48 vs 0.36). In the plot, the five groups could be separated distinctively, with Groups 1 and 5, and Groups 2 and 4 being closer to each other forming two opposite poles of the distribution, while the members of Group 3 were more dispersed in the III. and IV. quarters of the graph.

The five groups significantly differed from each other in the effects of the main variance components (genotype and water) and in the average values of the various protein composition traits, which are listed in Table 4. The difference between the groups was the largest in the epitope content, followed by the polymer and avenin contents. Group 1 was characterized by the lowest avenin and epitope content and by the highest polymer content, which was more pronounced in the rain-fed treatment. Group 5 was the second in all these traits. In addition, these two groups were the most stable across the two treatments, their protein parameters were not influenced or only to a very small extent by the additional water supply. On the other hand, Groups 2 and 4 had the highest protein content, and the lowest polymer content and they had the two highest epitope content, as well. This latter parameter was the worst for Group 4 cultivars. Of the groups, Cluster 2 was influenced by irrigation to the largest extent followed by Cluster 3, especially the polymer, avenin and epitope content of these two groups were primarily determined by the water and not by the genotypic differences. Cluster 1 had the most favorable combinations of different protein characteristics in a relatively stable manner and was more diverse in terms of variety origin. This group included genotypes from six of the eight countries, Germany, France, Hungary, Poland, the United Kingdom, and the United States.

4. Discussion

Oat is an ideal crop in certain agricultural regions because of its ability to thrive in the short seasons of cool and wet climates with long periods of daylight (Buerstmayr et al., 2007). It is a well-documented fact that changes in weather conditions significantly influence plant development, morphological and yield-related traits (Buerstmayr et al., 2007; Peltonen-Sainio et al., 2011; Klink et al., 2014; Chappell et al., 2017).

While the alteration of macroscopic factors such as grain yield, plant height and lodging severity influenced by growing conditions of oats is well demonstrated by the above-cited studies, our knowledge of the effects of growing conditions on the chemical composition as well as on nutritive and functional properties of oat grain is rather limited. The chemical composition of the oat hull is greatly influenced by weather variations during the oat growth phase (Schmitz et al., 2020), but no similar data is available on composition changes in the whole grain. Here, we examined the stability of protein composition properties of winter oats in relation to the excess water available during the grain-filling period.

4.1. Protein composition

The benefits of applications of oats as human food sources are directly related to protein composition. Protein composition is directly related to two important aspects of the end-use quality of oats. The ratio of polymeric and monomeric storage proteins in the samples is a key factor in determining important techno-functional characteristics such

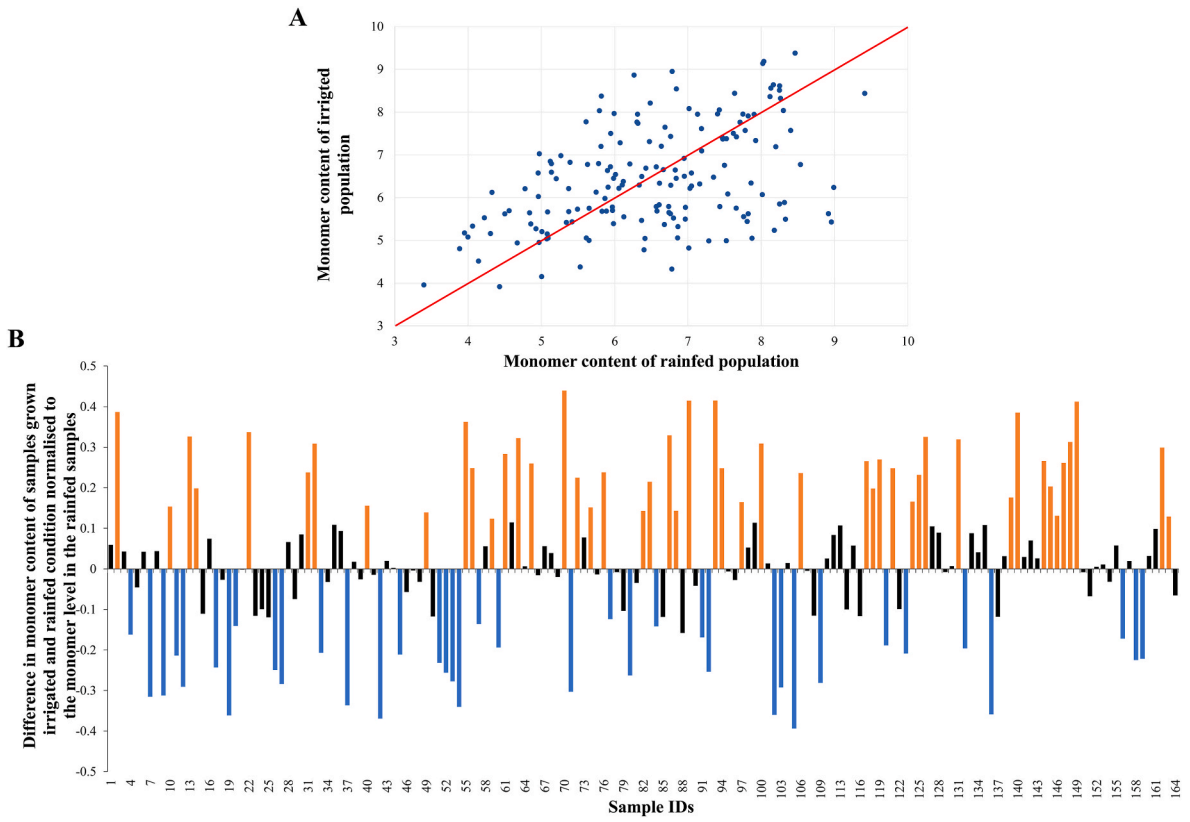

Fig. 4. The effect of irrigation on the monomer protein content of samples (% of total protein) grown under rainfed and irrigated conditions. (A) Comparison of monomer contents of samples. Data points above or under the 45-degree diagonal drawn in red indicate alteration caused by irrigation. (B) The difference in monomer protein content between samples grown under rainfed and irrigated conditions was normalized to the rainfed data [$\mathbf{d}_{\mathbf{monomer}}$ = (IRR-RF)/RF]. Bars drawn in black represent no significant alteration; while orange and blue bars show significant positive or negative changes in monomer content where the absolute value of means of **d** relative difference calculated from the replicate measurements is three times larger than its standard deviation. Detailed values are shown in Supplementary Table 2. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

as emulsifying and foaming properties, which play an important role in the application of oats in different functional food products. The information on the genetic and environmental factors on the amount and composition of avenin polypeptides – containing celiac epitopes – can help to select safe cultivars suitable for a gluten-free diet.

To avoid any risk of losing benefits caused by the alteration of protein composition as a result of changes in weather conditions, it is important to improve our knowledge about the relationships between various growing conditions and the protein composition of oat.

In this research, the well-established SE-HPLC method developed originally for analyzing wheat proteins has been adapted to analyze the distribution of the total protein content of rainfed and irrigated samples by a one-step extraction procedure followed by size-based separation.

Size exclusion liquid chromatography (SE-HPLC) is a historical technique widely employed for the detailed characterization of therapeutic and food proteins and can be considered as a reference and powerful technique for the qualitative and quantitative evaluation of aggregates. The main advantage of this approach is the mild mobile phase conditions that permit the characterization of proteins with minimal impact on the conformational structure and local environment (Fekete et al., 2014).

SE-HPLC is a powerful technique for size-based separation of proteins providing quantitative size distribution information. Since its first application in cereal science (Batey et al., 1991; Gupta et al., 1993) the methodology plays an important role in relating techno-functional properties to the protein composition of wheat, barley and rye (Janes and Skerritt, 1993; Nilsson, 2009; Wrigley et al., 2006; Van Der Borght et al., 2006; Silva et al., 2008; Békés, 2012; Redan et al., 2017) but legumes, for example, soybean (Oomah et al., 1994) and cereal-soybean blends (Maforimbo et al., 2006; Lamacchia et al., 2010).

Interestingly, no application of SE-HPLC for characterizing oat proteins is reported in the critical work of Sunilkumar and Tareke (2017) reviewing the analytical methods for measurement of oat proteins by covering 2000 works published between 1970 and 2015. The technique in our study has been applied not only for the quantitative analyses of the different protein fractions of the samples – determining the relative distribution of polymeric and monomeric globular proteins and avenins in the total protein content of the samples – but it was possible to convert the RP-HPLC based avenin compositional data and toxic epitope content of the different avenin polypeptides to [mg/100 g sample] unit by using the avenin % derived from the SE-HPLC analysis.

In the light of results shown in Tables 1 and 2, large inter-cultivar variation has been demonstrated in protein content and composition among samples grown under rainfed and irrigated conditions. The alterations of protein expression caused by the irrigation did not show a uniform pattern: protein content or any protein parameter, investigated could show a significant increase in the irrigated sample and a significant drop in the case of another cultivar. The relative differences of parameters observed in the samples grown under the two conditions, $\mathbf{d}_{\mathbf{x}}$ was introduced to monitor variation among varieties. Using this parameter, the cultivars can be ranked and varieties showing extreme responses to irrigation were identified. Because of the fact that the relative differences are calculated from the replicate measurements of two samples, the Gaussian error propagation law (Skoog et al., 2007) was applied to the statistical analysis of determining the significant alterations of mean values for the different cultivars.

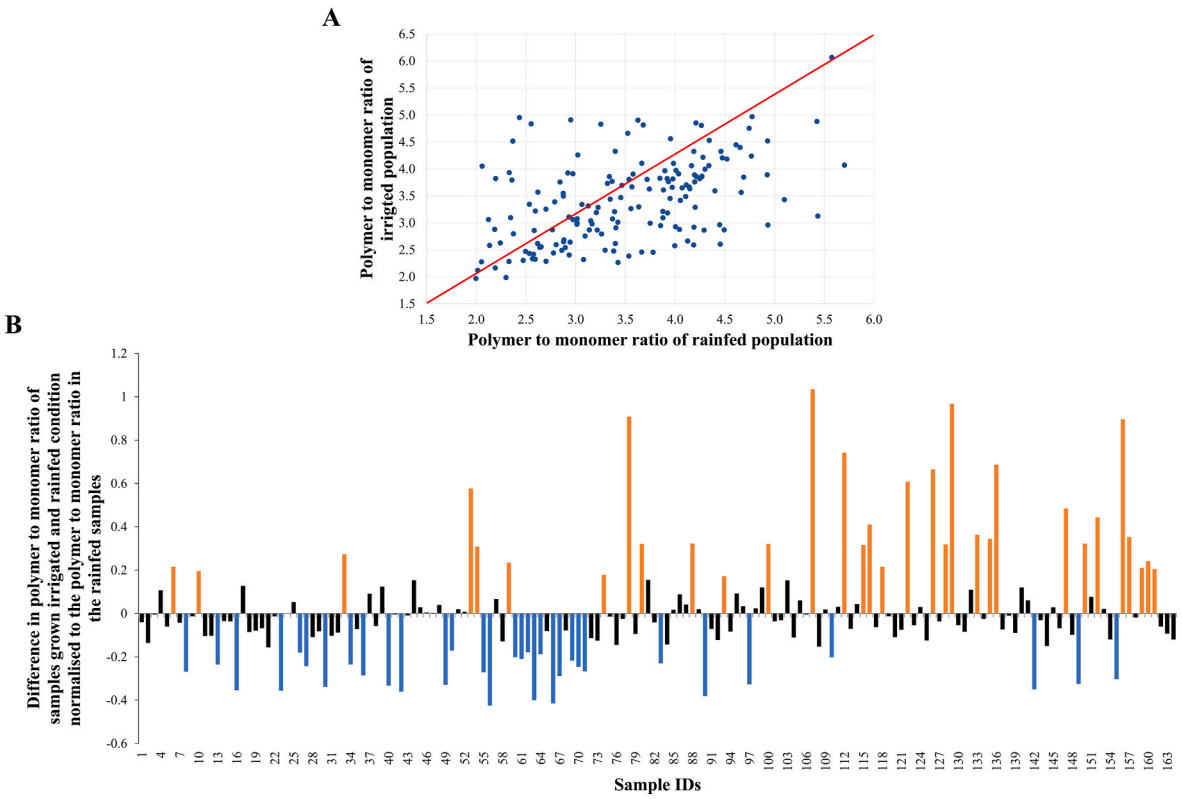

Fig. 5. The effect of irrigation on the polymer/monomer protein ratio in samples grown under rainfed and irrigated conditions. A) Comparison of polymer/monomer contents. Data points above or under the 45-degree diagonal drawn in red indicate alteration caused by irrigation. (B) The difference in polymer/monomer ratio between samples grown under rainfed and irrigated conditions was normalized to the rainfed data [$\mathbf{d}_{\mathbf{ratio}}$ = (IRR-RF)/RF]. Bars drawn in black represent no significant alteration; while orange and blue bars show significant positive or negative changes in polymer to monomer ratio where the absolute value of means of **d** relative difference calculated from the replicate measurements is three times larger than its standard deviation. Detailed values are shown in Supplementary Table 2. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Two-way ANOVA on cultivars and treatments investigating the effects of additional water supply on the protein content and composition as well as epitope content of the 164 oat samples.

	Cultivar		Treatment		Cultivar x treatment	
	F	p	F	p	F	p
PROTEIN	27.80	0.00	4.60	0.00	3.20	0.00
POLYMER	49.70	0.00	10.20	0.00	4.50	0.00
MONOMER	324.70	0.00	55.60	0.00	17.20	0.00
POLYMER to MONOMER ratio	249.80	0.00	61.80	0.00	21.30	0.00
EPITOPE content	1756.00	0.00	738.00	0.00	114.00	0.00

4.2. Oat proteins and CD

The safe consumption of oats for patients with celiac disease required that the product is uncontaminated with wheat, barley or rye, and specially produced to avoid gluten contamination above 20 ppm, which is the internationally determined and agreed threshold.

Hardy et al. (2015) clarified the evidence that ingestion of oats activates avenin-specific T cells in patients with celiac disease.

T cells are re-activated by barley *in vivo* and a family of deamidated hordein peptides related to DQ2.5-hor-3 epitope that are largely responsible for T cells activated by structurally related avenin sequences. They observed no avenin cross-reactivity by T cells induced following oral wheat challenge *in vivo*, and found that only hordein-specific TCC, and not wheat gliadin-specific TCC cross-reacted with avenin. This study also reported that 8% of HLA-DQ2.5+ celiac patients mobilize T cells specific for a family of avenin peptides, including DQ2.5-ave-1a and DQ2.5-ave-1b after 3 days of 100g oat intake (Alves et al., 2017). Their findings that avenin-reactive T cells are ubiquitous in HLA-DQ2.5+ CD patients, and that eating oats, and more so barley, activates this T cell population, helps explain the disparity between oats feeding studies and *in vitro* T cell studies.

They concluded in accordance with other oat feeding studies that because of the lack of intestinal damage or serological relapse and the widespread occurrence of the avenin-specific T cells in CD patients, the commonly consumed amounts are not toxic and oats should not be excluded from the diet in CD, but the safe dosage obviously depends on the individual's susceptibility.

The findings of Hardy and co-workers stand in contrast with previous results (Srinivasan et al., 1996; Hardman et al., 1997; Reunala et al., 1998; Hoffenberg et al., 2000; Janatuinen et al., 2000, 2002; Storsrud et al., 2003; Arentz-Hansen et al., 2004; Hogberg et al., 2004; Peraaho et al., 2004; Holm et al., 2006; Kemppainen et al., 2007; Koskinen et al., 2009; Sey et al., 2011; Cooper et al., 2013; Kaukinen et al., 2013; Atanasova et al., 2013), which shows definite relapse of celiac patients with uncontaminated oat intake (15–100g) for 3–24 months.

In summary, reactivated immune response due to substantial oat intake was quantitatively less and qualitatively different, compared to the approximately 5–10 g of gluten derived from wheat barley or rye (Tye-Din et al., 2010).

In the study of Leišová-Svobodová et al. (2022), genes for potentially harmful avenins, globulins and α-amylase/trypsin inhibitors were investigated using PacBio sequencing technology of enriched libraries. The research group concluded, that whether oats are less toxic to people with celiac disease is a question of dose. Thus, patients with celiac disease, depending on their individual susceptibility, could consume oats

Table 3
The significance of the effects of irrigation and a list of samples with extreme effects caused by irrigation on the protein content and composition as well as epitope content of the 164 oat samples.

		Protein	Polymer	Avenin	Monomer	Polymer/Avenin	Epitope
$[abs[mean(d_x)]_{crit}$ **		0.035	0.065	0.160	0.121	0.158	0.320
Significant negative effect (IRRIG < RAINFED)	n	31	21	22	39	32	16
	%	18.90	12.80	13.41	23.78	19.51	9.76
No significant effect (IRRIG ~ RAINFED)	n	115	121	107	78	100	119
	%	70.12	73.78	65.24	47.56	60.98	72.56
Significant positive effect (IRRIG >RAINFED)	n	18	22	35	47	32	29
	%	10.98	13.41	21.34	28.66	19.51	17.68
Samples with EXTREME negative d_x values		137* (−12)+	90 (−12)	107 (−0.41)	110 (−0.39)	56 (−0.42)	60 (−0.50)
		40 (−0.09)	65 (−0.12)	129 (−0.39)	45 (0.37)	66 (−0.42)	122 (−0.49)
		149(0.09)	62 (−0.12)	156 (−0.38)	19 (−36)	63 (−0.40)	15 (−0.46)
		89 (−0.07)	149 (−0.12)	78 (−0.38)	106 (−036)	90 (−0.38)	129 (−0.46)
Samples with EXTREME positive d_x values		138 (0.05)	107 (0.20)	54 (0.57)	74 (0.44)	107 (1.03)	149 (0.60)
		133 (0.05)	129 (0.19)	65 (0.51)	97 (0.42)	129 (0.97)	76 (0.58)
		113 (0.05)	78 (0.19)	62 (0.47)	93 (0.41)	78 (0.91)	82 (0.54)
		148 (0.05)	136 (0.17)	43 (0.47)	156(0.41)	156 (0.89)	14 (0.54)

** - [abs[mean(dx)]crit – the critical value of dx relative difference of parameters in the samples grown under IRR and RF conditions above which the alteration of the parameter is significant ($p<0.05$).
* - Sample code, + - dx.

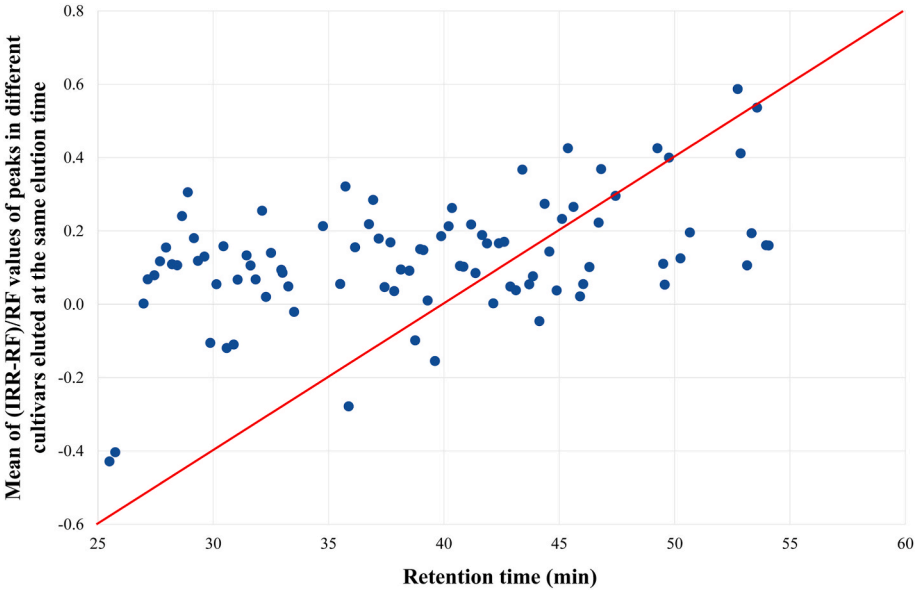

Fig. 6. The relationship between the elution time of avenin proteins and the means of the relative difference values for avenin contents between samples grown at irrigated and rainfed conditions eluting at a given elution time using RF-HPLC separation.

more safely.

Kamal et al. (2022) carried out a proteogenomic investigation of gene families related to human health and nutrition in oats. The results showed that only a subset of encoded avenin proteins contain coeliac disease-associated immune-reactive regions compared with the high prevalence found in wheat or barley. In addition, the low copy number of genes encoding coeliac disease epitopes, low frequency of detected T cell epitopes in the protein sequence, low occurrence of other highly immunogenic proteins, and the proportion of avenins within total oat protein and relative immunogenicity of avenin epitopes, all support the inclusion of oats in gluten-free diets.

Vriz et al. (2021) made a ranking of immunodominant epitopes in celiac disease and identified reliable parameters for the safety assessment of food proteins, including oat avenins. The study highlighted that a positive relationship has been identified between the number of prolines and the risk of gluten T cell epitopes. The overall ranking showed in decreasing order of immunological relevance: α-gliadins > ω-gliadins > hordeins > γ-gliadins ~ avenins ~ secalins > glutenins.

In this study the cumulative amounts of the presumably immune reactive avenin proteins in the samples were determined and expressed as a percentage of the sample mass by combining the peak data of RP- and SE-HPLC separation and protein content of the samples. The results proved, that the effect of irrigation on the epitope level does depend on the composition of the avenin fraction, and the alteration of expression levels of these avenin polypeptides. The latter is determined by using three independent analytical data.

Toxic epitopes were determined by summing up the relative amounts of RP-HPLC separated avenin polypeptides, containing epitopes, selected by their mobility data in comparison with the proteomic data of Tanner et al. (2019), followed by the conversion of these data to mg/100 g sample dimension, by the use of the protein content and the SE-HPLC based avenin content of the samples. Using Tanner's proteomics data in this way, it is assumed that their data - which are based on the detailed study of a single cultivar (cv. Wandering) - is representative for oat cultivars in general. The approach for the prediction of epitopes from RP-HPLC data is strictly reliable when data is supported and

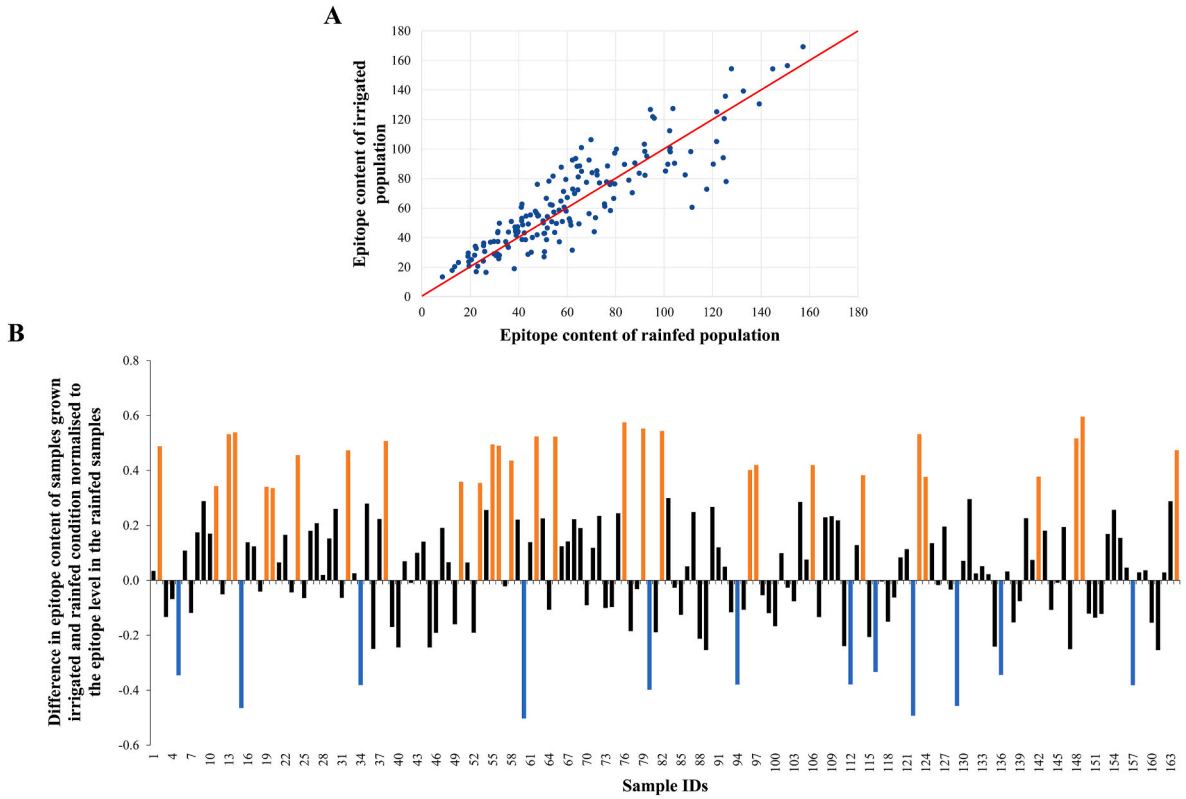

Fig. 7. The effect of irrigation on the epitope content of samples (mg % sample) grown under rainfed and irrigated conditions. A) Comparison of epitope contents. Data points above or under the 45-degree diagonal drawn in red indicate alteration caused by irrigation. (B) The difference in epitope content between samples grown under rainfed and irrigated conditions was normalized to the rainfed data [$\mathbf{d}_{\mathbf{epitope}}$ = (IRR-RF)/RF]. Bars drawn in black represent no significant alteration; while orange and blue bars show significant positive or negative changes in epitope content (mg/100 g sample), where the absolute value of means of **d** relative difference calculated from the replicate measurements is three times larger than its standard deviation. Detailed values are shown in Supplementary Table 2. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

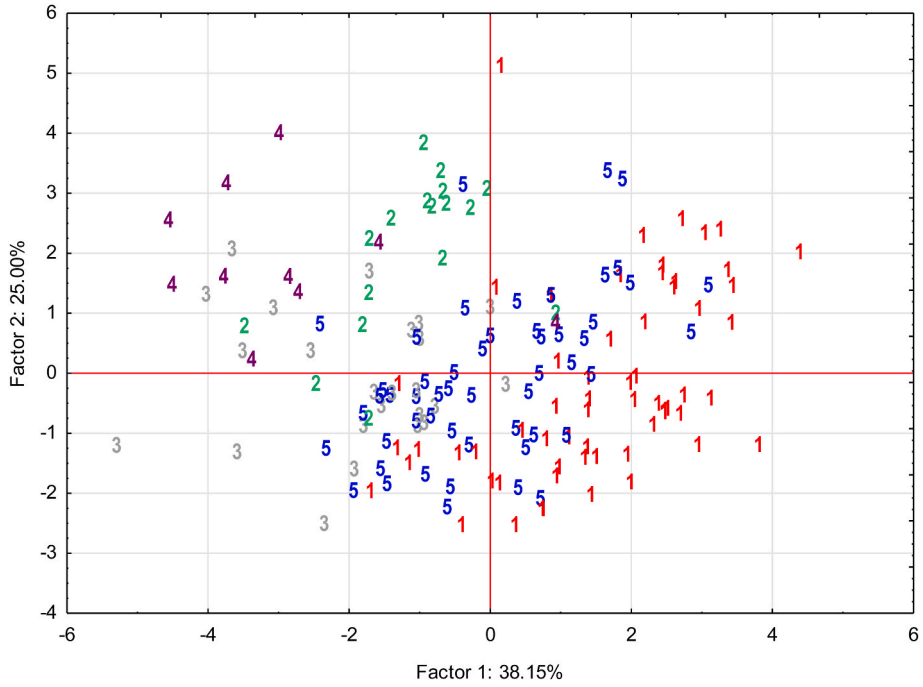

Fig. 8. Quality-based distribution patterns of the 164 winter oat genotypes visualized via Principal component analysis carried out on the phenotypic data-matrix of various quality traits from both the rain-fed and irrigated treatments (Genotypes are labeled with their cluster positions established via K-mean clustering and Discriminant analyses).

Table 4
Characterizations of the phenotypic clusters of winter oat genotypes via within-cluster ANOVA and with the comparison of the cluster average values of the various quality traits in rainfed and irrigated treatments.

Trait	Factor SS%/P level[1]	Cluster 1 n = 61	Cluster 2 n = 17	Cluster 3 n = 24	Cluster 4 n = 10	Cluster 5 n = 52
Protein	Genotype	97.2***	89.4***	97.4***	94.4***	94.1***
	Water	0.0^{ns}	2.6*	0.3^{ns}	0.0^{ns}	0.2^{ns}
Clu_ave_RF[2]	0.0001	13.3b	14.5a	13.2b	14.1a	13.2b
Clu_ave_IR	0.0024	13.2b	14.2a	13.3b	14.1a	13.1b
RF – IR[3]		0.05^{ns}	0.33*	−0.11^{ns}	−0.02^{ns}	0.08^{ns}
Polymer	Genotype	74.7***	34.4ns	68.7*	83.4*	71.1***
	Water	5.0***	42.9***	11.6**	0.2^{ns}	0.1^{ns}
Clu_ave_RF	1.61E-10	74.7a	68.5c	69.8c	68.6c	71.8b
Clu_ave_IR	3.77E-11	73.1 ab	74.0a	67.2c	68.3c	72.0b
RF – IR		1.64***	−5.54***	2.65**	0.35^{ns}	−0.21^{ns}
Monomer	Genotype	75.5**	57.9*	69.1*	62.3*	68.6*
	Water	1.7*	16.4**	0.5^{ns}	0.2^{ns}	0.0^{ns}
Clu_ave_RF	1.73E-08	5.8e	7.6 ab	7.0bc	6.5de	6.7cd
Clu_ave_IR	0.0330	6.1b	6.5 ab	6.8a	6.4 ab	6.7 ab
RF- IR		−0.29*	1.07**	0.15ns	0.08^{ns}	0.04^{ns}
Avenin	Genotype	71.8***	39.1ns	74.8***	82.5*	72.2***
	Water	4.5**	33.4***	12.4***	0.4^{ns}	0.1^{ns}
Clu_ave_RF	1.30E-07	19.5c	23.9a	23.2a	24.9b	21.5b
Clu_ave_IR	1.8E-11	20.8b	19.5b	26.0a	25.3a	21.3b
RF – IR		−1.35**	4.47***	−2.80***	−0.43^{ns}	0.17^{ns}
Epitope	Genotype	80.3***	41.8ns	54.0**	82.0**	65.0*
	Water	0.9^{ns}	33.4***	28.0***	7.2*	0.7^{ns}
Clu_ave_RF	1.50E-68	33.6e	106.4b	80.1c	132.9a	59.8d
Clu_ave_IR	1.53E-62	35.7e	87.2c	95.5b	141.3a	61.8d
RF – IR		−2.08^{ns}	19.14***	−15.44***	−8.49*	−1.97^{ns}

[1]% of variance explained by the two main factors within each cluster / the significance level of the difference between the average values of the five clusters.
[2]The average values of the five clusters followed by the same letters within one row are not significantly different from each other at P= 0.05 level, based on two-sample T-test.
[3]Column-wise difference and significance in the average values of rainfed and irrigated samples within each cluster based on paired two-sample T-test.

confirmed by amino acid sequence data demonstrating (at least in a representative number of cultivars) the actual presence and amounts of intact avenin epitope sequences in the distinguished RP-HPLC peaks.

Because of the limited resolution of the RP-HPLC separation of avenin proteins, some oat polypeptides co-elute producing false-positive results. Therefore, the predicted epitope levels have to be interpreted as upper limits, a measure of the possible variation of epitope contents in the cultivars in the sample population rather than the exact epitope levels in the individual samples.

The most important observation in this study is that toxic epitopes were detected in each cultivar. However, the large variation in epitope levels allows us to believe that the amount of the potentially harmful components can be reduced by careful selection during breeding and by adequate agronomic techniques such as a proper level of irrigation.

A large inter-cultivar variation in epitope level in RF samples has been found in this study: (min: 8.83, max: 157.36), which was echoed in a significantly increased manner in the IRR samples (min: 13.44, max: 169.36) (Table 1). The relationship between the epitope content data in the RF and IRR samples (r = 0.886) is much stronger than found for avenin composition (r = 0.501) (Table 1). This is partly realized by comparing the RP-HPLC-derived data on the avenin proteins in the two RF and IRR) populations (Fig. 6). Qualitatively the corresponding samples are identical in the RF end IRR samples for each cultivar (the same polypeptides can be identified in each of them). Quantitatively, however, a slight increase in the amounts caused by irrigation can be observed, which is more dominant in the cases of more hydrophobic avenin proteins. The extent of the alteration of epitope levels was not significant in almost three quarters of the cultivars (n = 119 from 164), a significant drop was detected in 16 cases (9.76%) while irrigation increased the epitope levels in 29 cultivars (17.685) (Table 3, Fig. 7). The list of cultivars with extremely negative and positive effects shown also in Table 3 could be utilized in breeding situations aiming for new oat cultivars with lower epitope levels.

Hardy et al. (2015) concluded in accordance with other oat feeding studies that because of the lack of intestinal damage or serological relapse and the widespread occurrence of the avenin-specific T cells in CD patients, the commonly consumed amounts are not toxic and oats should not be excluded from CD diets, but the safe dosage obviously depends on the individual's susceptibility.

However, the lower the epitope level, the better. Our results have proven that in spite of the significance of the environmental factors, such as the amount of water available, the effect of genotype was a highly significant component of all the protein properties studied. We have shown that in this large collection of winter oat genotypes originating from different countries, there is a significant variation in protein composition as well as epitope content. These facts represent a strong basis for identifying genotypes with specific protein composition and for carrying out crossing and breeding schemes directed to a specific improvement of newly released oat cultivars.

As it is indicated in Table 3, significant positive or negative irrigation-based alteration in protein composition was detected in around one-third of the cultivars. The largest variation is shown in the monomer fraction: a decrease in the relative amount of these proteins in 39 and an increase in 47 cultivars. These values are 21 and 22 for the polymeric fraction and 22 and 35 for the avenins, while the overall protein content altered negatively in 31 cases and increased in 18 cases. An identical number of cultivars (n = 32) with positively and negatively significant changes in the ratio of polymeric to monomeric fraction has been detected, all together close to 40% of the whole population. It became also possible to group the cultivars based on their protein compositions and on their reactions to irrigation. The comparative grouping revealed several facts. It can be stated that a low level of epitope content is associated in general with low protein and avenin, but with high polymer content. Irrigation had the strongest effect on the epitope, polymer and avenin levels, while protein content was influenced to the smallest extent. These effects were, however, highly genotype-dependent and groups of oat genotypes with specific water reactions could be clearly differentiated. The higher the avenin and epitope content, the stronger the reaction to the amount of water. What is important to emphasize, based on our results, is that genotypes with

low epitope levels were the most stable under the various water regimes. Their epitope content was not affected by water availability.

Ranking the cultivars based on their $\mathbf{d_x}$ values for a certain protein parameter and their cluster positions allowed the selection of germplasm to be used in breeding programs in order to develop new varieties with a given purpose. The list of cultivars with the four most extreme levels of alteration caused by the excess of water is tabulated in Table 3 and their cluster position in Supplementary Table 1.

5. Conclusion

In studying the protein composition in a large collection of winter oat genotypes, great extents of variation were identified in the various parameters and the significance of the genotypic effects in determining them was proven. In addition, it was also established that irrigation during grain filling significantly affected the various quantitative parameters of protein composition, but not its qualitative structure. This environmental effect, however, was again strongly genotype-dependent. Winter oat genotypes with low levels of avenin epitope content were identified and it was proven that this characteristic was independent of the environmental factor of water availability. Altogether 15 of the 164 winter oats were identified as a very low epitope-containing genotype irrespective of the environmental circumstances (<30 mg epitope/100 g sample). The most promising genotypes originated from four geographic areas, two from the UK (samples 12 and 14), three from DEU (samples 45, 48, and 163), four from the USA (samples 94, 102, 106, and 123), and six from Hungary (sample 55, 58, 63, 68, 76 and 144) are appropriate to initiate a specific breeding program for CD patients.

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CRediT authorship contribution statement

Gyöngyvér Gell: Designed the study, Performed experiments and analyzed data, Wrote the manuscript. **Ildikó Karsai:** Designed the study, Providing samples and sample preparation, Performed experiments and analyzed data, Wrote the manuscript. **Zita Berki:** Providing samples and sample preparation, Performed experiments and analyzed data. **Ádám Horváth:** Providing samples and sample preparation, Performed experiments and analyzed data. **Christakis George Florides:** Performed experiments and analyzed data. **Zsófia Birinyi:** Performed experiments and analyzed data, Wrote the manuscript. **Dalma Nagy-Réder:** Performed experiments and analyzed data, Wrote the manuscript. **Balázs Varga:** Providing samples and sample preparation, Performed experiments and analyzed data. **András Cseh:** Performed experiments and analyzed data, Wrote the manuscript, input from all authors. **Ferenc Békés:** Designed the study, Performed experiments and analyzed data, Wrote the manuscript. **Ottó Veisz:** Designed the study, Providing samples and sample preparation, Wrote the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ferenc Békés, co-author is the owner of the FBFD PTY Ltd., Sydney, Australia, which has no participation or any financial benefit from this work.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2022.10.032.

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