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Identification of perspective oat cultivars with a minimum content of gluten homologous peptides

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Abstract

There is still an ambiguous opinion on oat regarding its safety for people with celiac disease (CD). Some studies have confirmed different content of oat immunoreactive epitopes in different cultivars while others explain the differences in consequence of cross-contamination with gluten-rich species or as ELISA cross-reactivity of oat homological epitopes with antibodies against wheat gliadin. Our study was based on a two-year mapping of oat immunoreactive epitopes in a set of 132 oat cultivars using the G12-based ELISA kit. Although repeated evaluation confirmed high interannual variability (RSD ≥ 30%) in approximately 2/3 of the cultivars, the permitted gluten content (20 mg kg^{-1}) has not been exceeded except for contaminated cultivar Sirene. The polymorphism of purified avenins determined by SDS-PAGE revealed the occurrence of 2 bands around 30 kDa in oat cultivars with relatively high gluten content (12-16 mg kg^{-1}) except for the cultivar Leo while this pattern occurred only in 50% oat cultivars with low gluten content. Quantification of gluten epitopes on purified avenin unit further revealed the three materials (Mojacar, Maris Oberon, SG-K 16370) with the lowest gluten content and presence of one band at 30 kDa. The band pattern at 30 kDa thus represents a promising breeding marker.

Keywords: oat, cultivars, avenins, gluten epitopes, celiac disease, ELISA,

1. Introduction

Oat (*Avena* L.) is a monocotyledonous crop from the *Poaceae* family cultivated almost all over the world in various climatic regions. Although oat is still predominantly used as forage and livestock feed, the popularity of oat consumption in the human diet recognizes the increasing trend [1]. Oat grain is an important source of proteins, fat, vitamins, minerals (Fe and Ca), fibers as well as important bioactive compounds such as β glucans and avenanthramides [2]. Therefore, oat consumption is recommended for all age spectra of human population [3-4].

Nevertheless, there is still an ambiguous opinion on oat regarding the people with celiac disease (CD). CD is an autoimmune disease associated with a permanent intolerance to gluten with prevalence of about 1 % in population [5]. Currently, the only effective treatment is the abidance of gluten free diet (GFD) but the recommended amounts of fiber, iron and calcium can be more difficult to obtain. Thus, supplementing a GFD with oats could potentially diminish nutrient deficiency and may improve the quality of their life.

The above mentioned ambiguity is mainly connected with inconsistent outcomes observed in oat clinical studies some of which have mentioned that some CD patients possess sensitivity to oat proteins [3].

Oat prolamin proteins homologous to wheat glutenin and gliadin proteins containing gluten peptides are called avenins. In oat, two immunoreactive epitopes including DQ2.5-ave-1a „PYPEQQEPF“ and DQ2.5-ave-1b „PYPEQQQPF“ were identified in avenin molecules. A

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phylogenetic analysis of avenin sequences in 13 *Avena* species including twelve diploid and tetraploid *Avena* species and a hexaploid *Avena* cultivar Gigant carried out by Londono et al. [6] revealed that oat genotypes usually encode 7 to 10 avenin genes belonging to four avenin phylogenetic groups. Regarding avenin immunoreactivity, avenin group II contained the epitope DQ2.5-ave-1b „PYPEQQQPF“ and avenin group III contained the epitope DQ2.5-ave-1a „PYPEQQEPF“ while avenin genes from groups I and IV contained no T-cell immunoreactive epitopes. However, the authors reported that none of the oat species studied lacked group II or III avenins, i.e., they all possess at least one of the two avenin-specific T cell epitopes. However, it should be borne in mind that the genes present in a given genome can be expressed at differential rates resulting in different final protein levels; thus, the resulting levels of immunogenic proteins cannot be estimated from the presence of genes or gene copies in the genome due to various mechanisms regulating transcription and posttranscription processes. Therefore, several studies, e.g., Comino et al. [7], Real et al. [5], and others, reported genotypic differences in the content of immunoreactive avenins resulting in an identification of several oat genotypes with the final levels of immunoreactive avenins well below the threshold of 20 ppm which possibly indicates low levels of accumulation for immunoreactive avenins. For more details on genotypic differences in the content of immunoreactive avenins, see the review by Kosová et al. [8].

Antibodies used for detection of immunoreactive gluten peptides in oat include antibodies raised against gluten peptides derived from wheat gliadins, namely the 33-mer (p57-89) derived from α2-gliadin, such as G12 and R5 monoclonal antibodies which are used in ELISA tests for determination of immunoreactive gluten peptides not only in *Triticeae* cereals, but also in oat [9-11].

Fritz and Chen [3] further declared that this discordance can be connected with an easy cross-contamination of oat with wheat, barley or rye gluten. Their meta-analysis in 12 strictly selected clinical studies confirmed significant clinical symptoms but no morphological effect due to gluten occurrence in oat. The next recent clinical meta-analysis by Pinto-Sanchez et al. [4] did not bring any evidence that the addition of oats to a GFD adversely affects symptoms, histology, immunity, or serologic features of patients with celiac disease. Nevertheless, the authors add that another detailed clinical study is needed.

A different cultivar toxicity of oat for celiac patient is mentioned as a next theory explaining observed variability in clinical symptoms. Comino et al. [7] and continuing research of Real et. al. [5] confirmed different level of immunotoxicity in individual oat cultivars based on their genetically different amino acid compositions of isolated avenins. Also, Ballabio et al. [12] evaluated the cross-reactivity between avenins and gliadins by SDS-PAGE/Immunoblotting and ELISA methods in 36 oat cultivars. In most oat samples, the content of cross-reactive proteins measured by ELISA was below 20 ppm, but in a few varieties, it was above 80 ppm.

On the other hand, Gilissen et al. [13] disagree with the interpretation of the immunogenic responses of the G12 monoclonal antibodies (Anb) in the case of oat and detected immunochemical difference designate as misleading results. According to these authors, it is caused by cross-reactivity with some homological sequences in avenins which do not have to correspond with clinically verified toxic sequences. Currently, only two avenin sequences, which are perhaps recognized by G12 Anbs, are mentioned as resistant to trypsin and chymotrypsin digestion [6], but these most likely occur in every oat variety [13].

Finally, Lexhaller et al. [14] found out that different ELISA test kits yielded variable results in dependence on the source of gluten (wheat, rye, or barley) and the gluten fraction (prolamins or glutelins). They revealed that gluten content was up to sixfold overestimated in case of rye and up to sevenfold underestimated in the case of barley. It is evident that different gluten sources showed highly variable reactivity with specific kit antibodies. So, a proper

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ELISA kit selection, especially for unknown source of gluten, will be essential because underestimation of toxic gluten content can bring serious risks for CD patients [14].

According to the above mentioned papers, it is only possible to summarize that oat cultivars contain different level of peptide epitopes which are homologue to toxic wheat gluten and some epitopes showed different immunogenicity as well [5]. On the other hand, the concentration of these oat epitopes is 5 – 10 thousand times lower compared to wheat gluten and the content of oat epitopes did not exceed the limit for gluten-free products (20 ppm) in most tested cultivars. Quantification of gluten epitopes with a more exact method such as LC-MS seems to be very promising [15], but still limited because of high cereal protein polymorphism and incomplete gluten databases of oat reactive epitopes.

So, the current strategy on the basis of the immunochemical (ELISA) approach could be aimed at detection of the oat cultivars showing a minimum reactivity (minimum content of homological gluten epitopes). Such cultivars have already been identified e.g. by Ballabio et al [12] or Comino et al. [7] and it is possible to suppose that these cultivars will represent a smaller risk for celiac patients as well.

The major aim of the study was focused on the utilization of useful combination of electrophoretic and immunochemical analysis by ELISA tests in order to achieve a higher sensitivity for detection of gluten-like homologous epitopes under LOQ thresholds of commercial immunochemical kits (ca 4 - 5 ppm). The detection of these low gluten-like protein contents will be very useful in breeding of novel oat cultivars safe for celiacs. We worked with a set of 132 oat genotypes including both cultivars and novel breeding materials and used standard G12-based immunochemical kit to determine materials with contrasting levels of immunoreactive peptides. We used SDS-PAGE and chromatography to determine specific bands (peaks) which would enable us to differentiate between high gluten-like and low gluten-like oat materials. We also determined the content of immunoreactive peptides on a purified avenin unit by G12-based kit as a more sensitive tool for mutual oat cultivar differentiation.

2. Materials and Methods

2.1 Plant Material and Field Trials

Analyses were carried out in a wide set of 132 commercial oat cultivars originated from 21 countries including selected 5 new Czech breeding lines. According to taxonomical classification, the set included 100 cultivars of *Avena sativa L.*, 11 cultivars of *Avena sativa var. nigra* L. and 21 samples of *Avena nuda* L. (Tab. 1 and Supplementary Table 1). Materials were cultivated in small breeding plots (4.5 m^2) at breeding station Selgen a.s. – Krukanice in the Czech Republic during 2 years (2018 - 2019). Oat grains (min. 200 g) were dehulled (except naked cultivars) and milled with a laboratory grinder (IKA-Werke, Germany). The obtained meal was used for subsequent chemical analyses.

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Tab. 1 Classification of the tested set of oat cultivars according to their taxonomy and their state of origin

Classification	Category	Number of materials
Taxon	*A. sativa*	100
	A. nuda	21
	A. sativa var. nigra	11
State of origin	FIN	6
	CZE	46
	CAN	8
	RUS	5
	GER	17
	FRA	10
	GBR	8
	USA	7
	BEL	2
	POL	2
	AUT	6
	HUN	1
	EST	2
	IRL	2
	NOR	1
	SCG	1
	NLD	2
	ARG	2
	ITA	1
	SWE	2
	ROU	1

2.2 Chemical analyses

All chemicals and solvents used were of analytical grade. Chemical analyses included immunoassay tests of gluten content in oat grain and purified avenins, prediction of crude protein content in oat grain using NIR spectroscopic method, purification, electrophoretic analysis and chromatographic quantification of avenins.

2.2.1 Crude protein content

Crude protein content (CP) in oat grain was detected using Nicolet Antaris II Fourier transform spectrophotometer equipped with an interferometer (Thermo Fisher Scientific). Approximately 25 g of intact wheat grains was placed on the rotary sample-cup spinner, and 64 interferometer sub-scans in ranges from 10,000 to 4000 cm−1 (wavelength 1000–2500 nm) and with a resolution of 2 cm^{-1} (0.5 nm) were applied for the collection of each spectrum sample by means of the software Omnic 7.3 (Thermo Fisher Scientific). Parameters of the CP prediction model included: Range of calibration for CP (14 % – 21 %): Correlation coefficient of calibration (r = 0.98), Standard error of calibration (SEC = 0.25 %), Standard error of cross validation (SECV = 0.58 %), Standard error of prediction (SEP = 0.54 %). Processing of the collected spectra as well as CP prediction was carried out using TQ analyst® software (company, state).

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2.2.2 Immunoassay tests

Standard measurements in oat meal were performed by using an ELISA test kit AgraQuant Gluten G12 assay (Romer labs Diagnostics) according to the manufacturer´s protocol. All ELISA measurements were in a separate room to avoid gluten contaminations. The absorbance was recorded at 450 nm by using Sunrise (Tecan) microplate reader [11]. Four replications of assessment was used per sample.

A similar immunochemical procedure were applied in case of purified avenin in selected 12 oat cultivars with significant lower concentration of homological gluten epitopes ($gluten_{(G12)}$). The resulting concentration of gluten-like homologous peptides per avenin unit (ng mg^{-1}) was determined as a ratio between the concentration of reactive gluten epitopes determined by G12-based kit (ng . ml^{-1}) and a concentration of purified avenin (mg . ml^{-1}) determined by RP-HPLC (see below).

2.2.3 Isolaion of pure avenins

Chilly precipitation of avenins was carried out according to Tanner et al. [16] with some modifications. 0.5 g oat flour was extracted in 1,5ml of 50% (v/v) ethanol, vortexing regularly over 1 h, and centrifuging at 3000 g for 1 h. The 50% ethanol supernatants were chilled at 4 ºC overnight, centrifuge as above and pellets redissolved in 0,3 ml 60% (v/v) ethanol during 2 h incubation at room temperature.

2.2.4 Avenin quantification by RP-HPLC

The applied RP-HPLC method were carried out according to Gojković-Cvjetković et al. [17] with following modifications. *Contents and characterization of isolated avenins* were assessed in samples (10 µl) through reverse-phase high performance liquid chromatography (RP-HPLC) using a Waters 2965 apparatus with UV detector (210 nm) and 300 SB-C8 Zorbax Poroshell ™ column (75×2.1 mm, 5 µm particles) linked to a Zorba 300SB-C8 cartridge guard column (RocklandTechnologies, Inc.Newport, DE). The external calibration curve was constructed with PWG gliadin (0.5 to 5 mg . ml^{-1} in ethanol/water). To elute the avenins from the column, 0.1% trifluoroacetic acid (TFA) in deionized water (eluent B) and 0.1% trifluoroacetic acid (TFA) in acetonitrile (ACN) of HPLC purity (eluent A) were applied and mixed to produce CAN gradient as follows: 0-1 min 23% A; 1-2,5 min 23-30% A; 2,5-9,5 min 30-47% A; 9,5-11 min 47% A; 11-13 min 47-23% A; 13-14 min 23% A at 1,5 cm3/ min flow. Control of the chromatography and data quantitation was provided by Empower Bulid 1154 software.

2.2.5. Avenin identification by SDS-PAGE

Polymorphism of avenin peptides was studied in condition of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to Leammli [18] on 12,5 %, separating gels and 4,6% stacking gels in Thermo Scientific™ Owl™ Dual-Gel Vertical Electrophoresis Systems(US) unit . Extractable proteins have been diluted in the ratio1:1(v/v) with the sample buffer (0,055M Tris –HCL pH 6,8, 2% SDS, 40% glycerol, 1 % DTT, 0,0025% bromophfenol blue) and heated at 90 ºC for 5 min. After the run, the proteins were fixed for 30 min in 12% trichloroacetic acid and stained for 30 min with Coomassie Brilliant Blue R-250. Molecular weights of the polypeptides were estimated by using Thermo Scientific™ PageRuler™ Unstained Broad Range Protein Ladder

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2.3. Data analyses

All statistical analyses (histograms, Pearson's correlation, graphs constructed on basis of ANOVA and Tukey's test of significance) were carried out using statistical software Statistica 7.1 CZ. Inter-year content variability of homological gluten peptides (G12) for the individual oat cultivar was calculated as a relative standard deviation (RSD) according to formula: 100 . s / $\bar{x}$, where 's' was the inter-year standard deviation of the oat cultivar and $\bar{x}$ was a mean of gluten$_{(G12)}$ content from years 2018 and 2019.

3. Result and Discussion.

3.1. Contents and variability of immunnoreactive gluten peptides in the set of oat cultivars

Two-year mean values of immunoreactive gluten peptides and the total content of grain proteins in the set of 132 oat genotypes are given in Table 1. The mean content of immunoreactive gluten peptides in the whole sample set was 7 mg . kg^{-1}. None of the tested cultivars except for the French cultivar Sirene exceeded the 20 mg . kg^{-1} limit for gluten-free foods safe for celiacs [19]. Only the French cultivar Sirene from 2018 harvest revealed 200-300 mg . kg^{-1} of immunoreactive gluten content. However, in 2019 harvest, the immunoreactive gluten content in Sirene was only 9.5 mg . kg^{-1} thus we could expect a contamination with another gluten source for Sirene in 2018.

Tab. 2 Statistical comparisons between gluten$_{(G12)}$ and crude protein content in the tested set of 132 oat cultivars (2018 – 2019)

Parameter		Mean	Min	Max	St. Dev	RSD (%)
Gluten$_{(G12)}$ (mg.kg^{-1})		7.0	2.0	17.5	3.8	53.7
Crude protein (%)		18.7	15.4	22.3	1.3	6.8
Correlation coef. Gluten$_{(G12)}$ vs. Crude protein	-0.13					

*The detected mean value 309.4 mg.kg^{-1} of Sirene cultivar from 2018 was not included

The following distribution analysis in content of homological gluten peptides showed evidently similar year distributions (Fig. 1). The next distribution analysis of RSD further confirmed a high inter-year variability (Fig. 2). The highest frequency of immunoreactive gluten content was found for 5 – 10 mg . kg^{-1} gluten range and the frequency significantly decreased for both higher and lower values. However, it has to be noted that the values of mean, standard deviation and RSD in 0 - 5 mg . kg^{-1} range are significantly affected by the limit of quantification (LOQ = 4 mg . kg^{-1}) and the limit of detection (LOD = 2 mg . kg^{-1}) of the kit used for gluten detection (AACC Method 38-52.01). The obtained values of immunoreactive gluten lower than 4 mg . kg^{-1} could show a higher rate of analytical uncertainty. The values of immunoreactive gluten lower than LOD (5 cultivars in 2018 sampling and 9 cultivars in 2019 sampling) were automatically assigned to 2 mg . kg^{-1} detection limit which thus concealed actual variability and increased the mean in the given category including the calculated mean valid for the whole set of oat samples.

The total distribution of the individual inter-year variability (RSD) in the immunoreactive gluten content revealed a broad range of values up to 90% (Fig. 2). Only one third (45) of the tested cultivars revealed inter-year variability lower than 30%. The high inter-year variability in immunoreactive gluten peptides cannot be explained by variability in total grain protein content which revealed significantly lower inter-year variability (6.8% as average variability for the individual cultivars tested). The correlation coefficient for immunoreactive gluten

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content and the total grain protein content was very low and insignificant (r = -0.13 see Tab. 2).

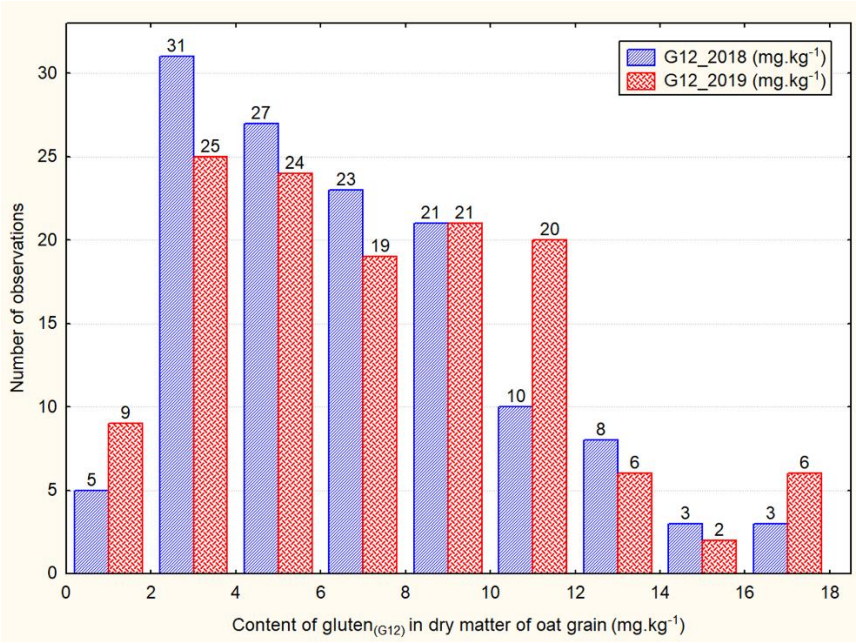

Fig. 1 Year distribution of detected content of reactive gluten$_{(G12)}$ in the set of 132 oat cultivars

*The detected mean value 309.4 ppm of Sirene cultivar from 2018 was not included

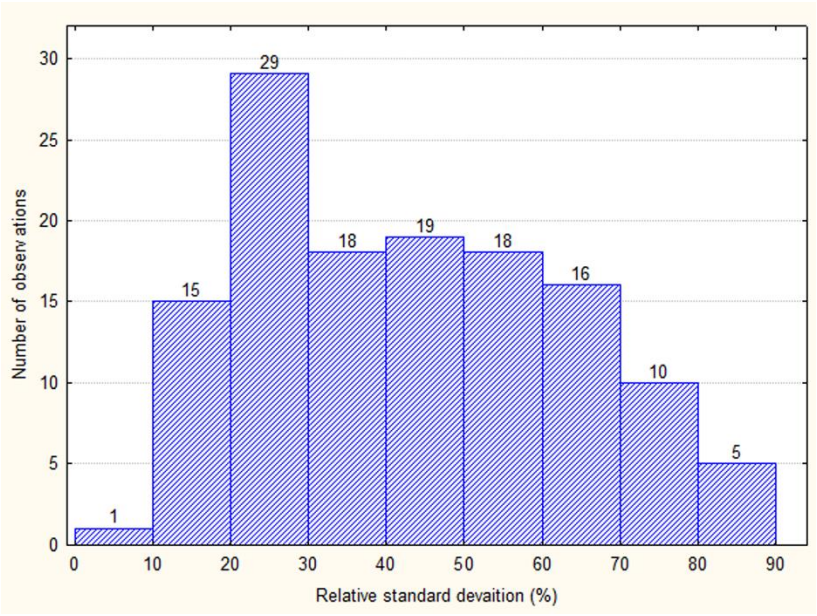

Fig. 2 Inter-year distribution of relative standard deviation (RSD) of gluten$_{(G12)}$ content in the set of 131 oat cultivars (2018 a 2019)

*The oat cultivar Sirene was not included

The high inter-year variability in immunoreactive gluten content in the majority of studied oat genotypes can be explained by combination of two major factors. The first one represent environmental conditions such as temperature stress (extreme temperatures) which affect the biosynthesis of different prolamins fractions and thus the abundance of avenins containing

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immunoreactive gluten-like sequences [20]. The other factor is associated with an identification of homologous gluten-like epitopes in avenins which are slightly different from /not identical with wheat gluten epitopes [6]. However, several avenin-derived epitopes revealed a significant cross-reactivity with wheat gluten epitopes. For example, avenin-derived sequence QPQLQ revealed significant cross-reactivity to wheat QPQLPY epitope while avenin-derived epitopes QPQQQA, QQQQPF, QPQQQA, QQQQPF, QPQQLP and QPQLPF revealed cross-reactivity to wheat gluten epitope QPQQPY.

Based on these findings, it should be more correct to speak only about detection of avenin-derived epitopes homologous to wheat gliadin 33-mer for whose detection the G12 monoclonal antibody was raised. Moreover, the avenin-derived epitopes determined in the present study do not correspond with avenin-derived epitopes reported to stimulate immune response in CD patients as reported by Tanner et al. [16]. It can thus be expected that non-contaminated oat samples with gluten-like immunoreactive peptide levels lower than 20 mg kg^{-1} will be safer for celiacs than analogous wheat-derived products with the same gluten contents.

3.2. Contrast groups of oat cultivars in content of immunoreactive gluten peptides

In the present study, we further focused on two groups of oat genotypes containing either ($\leq$ 5 mg . kg^{-1} or $\geq$ 11 mg . kg^{-1} of immunoreactive gluten peptides and revealing relatively low inter-year variability (RSD $\leq$ 30%). Statistical analysis confirmed a significant difference in the content of immunoreactive gluten epitopes detected by G12 monoclonal antibody ($Gluten_{(G12)}$) between the two groups (Fig. 3). In the group with gluten content higher than 11 mg . kg^{-1} which included 10 cultivars, significant genotypic and inter-year differences were determined as well. It was clearly indicated by non-overlapping confidence intervals among several oat cultivars in both tested years. In contrast, no significant genotypic and inter-year differences were determined in the group revealing low gluten content ($\leq$ 5 mg . kg^{-1}) which included 12 cultivars (Fig. 3).

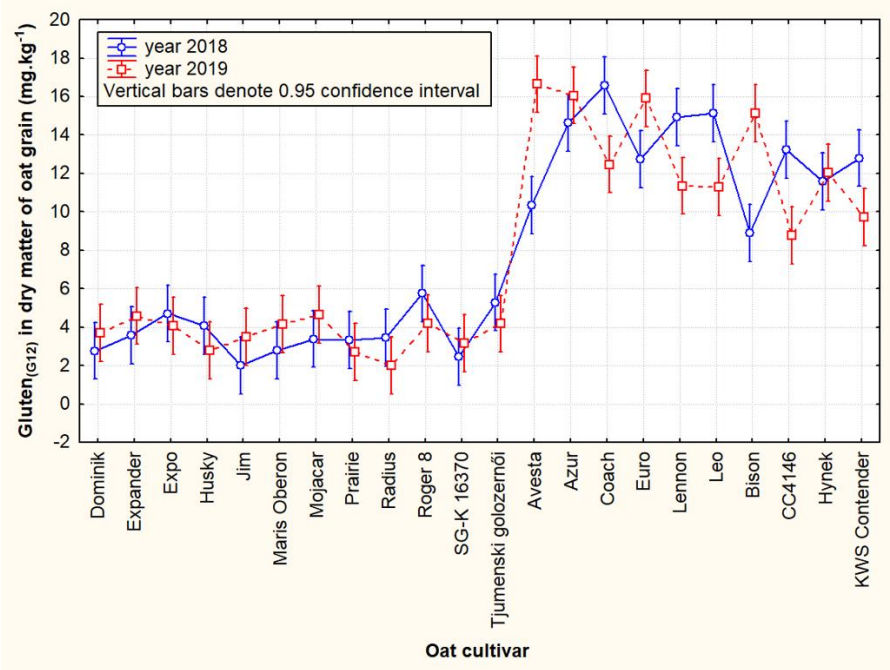

Fig. 3 Two-year statistical comparison of selected oat cultivars that showed lower inter-year variability (RSD $\leq$ 30 %) and contrast average gluten (G12) contents ($\leq$ 5 versus $\geq$ 11 mg . kg^{-1})

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Immunoreactive gluten contents around 16 mg . kg^{-1} which are close to the 20 mg kg^{-1} limit for celiacs were detected in 4 cultivars, but only in one year (Avesta - 2019, Azur – 2019, Coach – 2018 and Euro - 2019). With respect to the fact that the detected gluten-like reactive peptides reveal only sequence homology and cross-reactivity with wheat gluten peptides while clinical studies on the harmful effects of these avenin-derived gluten-like peptide homologs are still missing. It can be thus concluded that oat cultivars revealing gluten-like peptide contents lower than 20 mg kg^{-1} could be considered safe for CD patients except for some specific food products such as concentrated protein isolates. The approaches used for cereal grain protein isolation, the efficiency and utilization of extracted grain proteins are summarized in Hadnadev et al. [21]. Considering the generally lower nutritional quality of prolamin and glutelin fractions of cereals grain proteins [22], the producers of cereal grain protein concentrates will likely prefer isolation of only nutritionally richer albumin-globulin grain protein fraction where contamination with gluten-reactive peptides is very low.

3.3. Avenin protein polymorphism in immunoreactive contrast groups of oat cultivars

Resolution of oat grain protein fraction containing avenin fraction via SDS-PAGE and examples of purified avenin peaks detected by HPLC are provided in Fig. 4A and 4B. Consistent with the results of Tanner et al. [16], the significant 5 – 7 bands (peptides) in the range of 20-30 kDa were detected on electrophoretic gels. At 30 kDa where immunoreactive peptides were detected with G12 monoclonal antibody by Comino et al. [7], an important difference between two groups of oat genotypes associated with the presence of either a single band or double bands on the gels was detected. The group comprising oat cultivars with relatively higher gluten-like peptide content (≥ 11 mg . kg^{-1}) revealed double bands at 30 kDa in SDS-PAGE gels except for the cultivar Leo while in the group comprising oat cultivars with relatively low reactivity to G12, the frequency of the double band occurrence was only 50%, i.e., 6 out of 12 oat cultivars included in this group (Fig. 4A1 and 4A2). In addition, avenin polymorphism within different oat cultivars is only detected in SDS-PAGE gels which represents a useful tool for cultivar identification [23].

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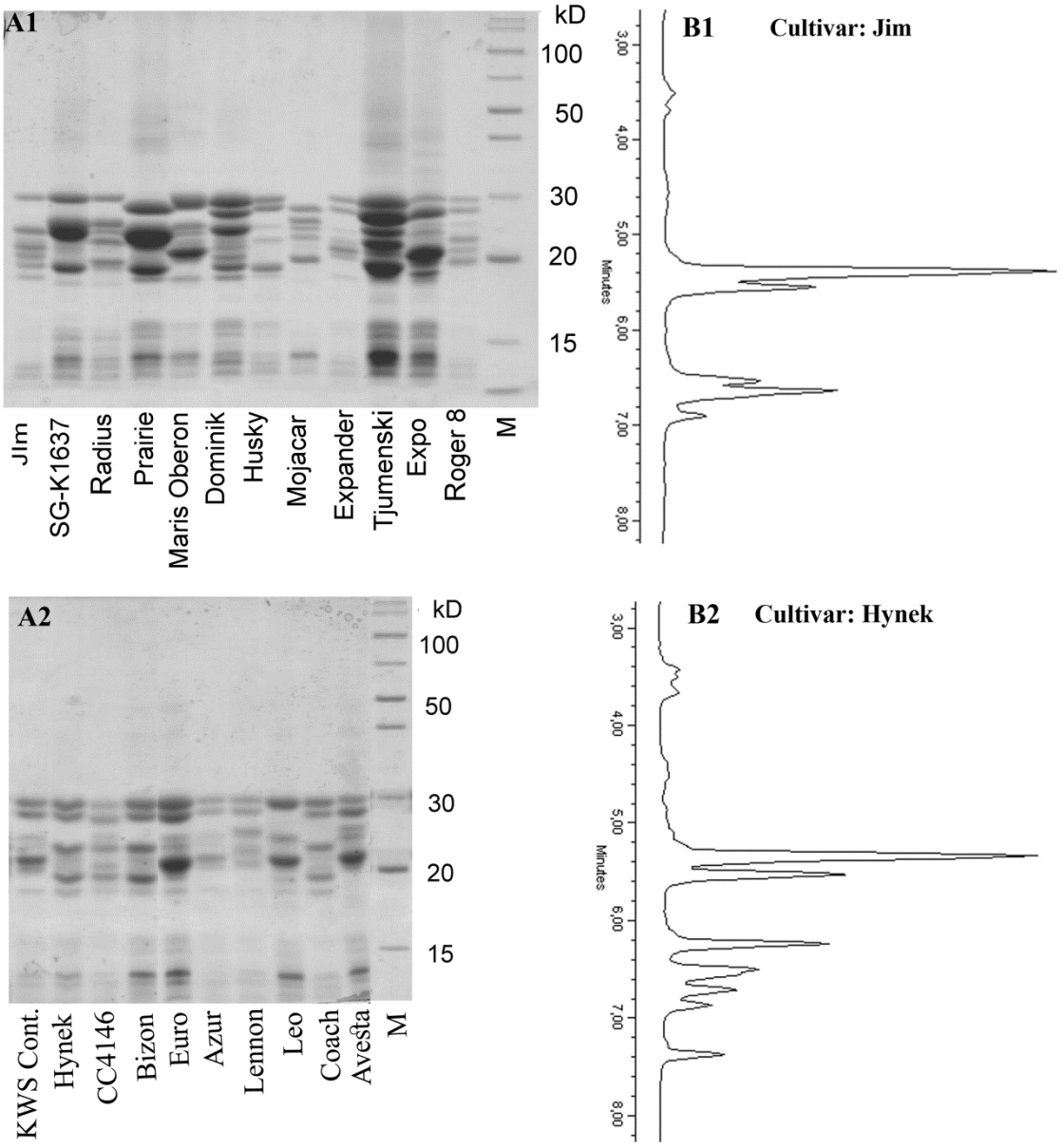

Fig. 4 Electrophoretic (A) – SDS PAGE and examples of chromatographic (B) - RP-HPLC visualization of isolated avenin extracts in 2 groups of oat cultivars with a lower (1) and a higher gluten$_{(G12)}$ contents (2).

The chromatographic separation (RP-HPLC), predominantly applied for avenin quantification, separated avenin peptides into two major groups of peaks (Fig. 4B1 and 4B2) corresponding to the results presented by Real et al. [5] and Tanner et al. [16]. Simultaneously, Real et al. [5] confirmed a close similarity of retention times between α (earlier eluted) and γ (later eluted) wheat gliadins and both separated group of avenins. However, ours as well as the spectrum of later eluted avenins in case of Real at al. [5] showed only a half of peaks (3 - 6) in comparison to the results published by Tanner et al. [16] who identified 5 major and 5 minor peaks in purified avenin fraction.

Compared to electrophoretic analysis, no clear differences between the two oat groups differing in the content of gluten-like reactive peptides were detected in HPLC spectra. Within the oat group revealing low gluten reactivity, the number of later eluted peaks

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assigned to γ avenin protein fraction was lower (3-4 peaks) in Jim, SG-K 16370, Maris Oberon, Husky and Mojacar cultivars. In addition, the peak eluted at 7.5 min was missing in this group while it was always present in the HPLC spectra from cultivars belonging to higher reactivity group (Fig. 4B2). These results thus indicate that mainly electrophoretic approaches could significantly contribute to the identification of oat genotypes with lower levels of gluten-like immunoreactive peptides.

3.4. Oat cultivars with minimum immunoreactive peptide contents per avenin unit

Our effort further focused on more precise interpretation of immunoreactive peptide content differences in case of mutually insignificant 12 oat cultivars values of which were often lower than the detection limit (LOQ 4 mg . kg^{-1}). Therefore, we tried to directly isolate and concentrate the pure avenin fraction (see 2.2.3). The efficiency of avenin isolation per one unit of grain dry weight was roughly by 25 - 50% lower in our study when compared to the results published by Tanner et al. [16]. These differences between our results and those published by Tanner et al. [16] could be associated with different genotypes analyzed in both studies as well as with different quantification methods used based on wheat gliadin standard.

The obtained results related to the contents of reactive gluten peptides expressed per unit of avenins led to an identification of significant varietal differences within the oat group with low content of gluten-reactive peptides (≤ 5 mg . kg^{-1}) but also relatively high inter-year stability. Considering roughly 1% avenin content in the total dry grain weight and the level of reactive gluten-like epitopes as 2-4 mg kg^{-1} in grain dry weight within the studied group of cultivars (Fig. 3), the resulting levels of gluten-reactive peptides should range around 200 - 400 ng . mg^{-1} per unit of avenin proteins. Contrary to these calculations, our results indicate 10 - 20 times lower levels of immunoreactive avenins ranging from 4 ng mg^{-1} (Mojacar) to 17.5 ng.mg^{-1} (Expo) (Fig. 5). Nevertheless, our results were not unrealistic and corresponded with the findings of Comino et al. [7] who also found a large variability in the level of immunoreactive epitopes per avenin unit (< 5.4 - 1340 ng . mg^{-1}). The cultivars Mojacar (4 ng . mg^{-1}), SG-K 16370 (5.2 ng . mg^{-1}) and Maris Oberon (5.7 ng . mg^{-1}) thus revealed the lowest content of immunoreactive gluten-like epitopes per unit of avenin proteins as compared to other oat cultivars from the low gluten-like reactivity group such as Dominik, Husky or Expo (Fig. 5).

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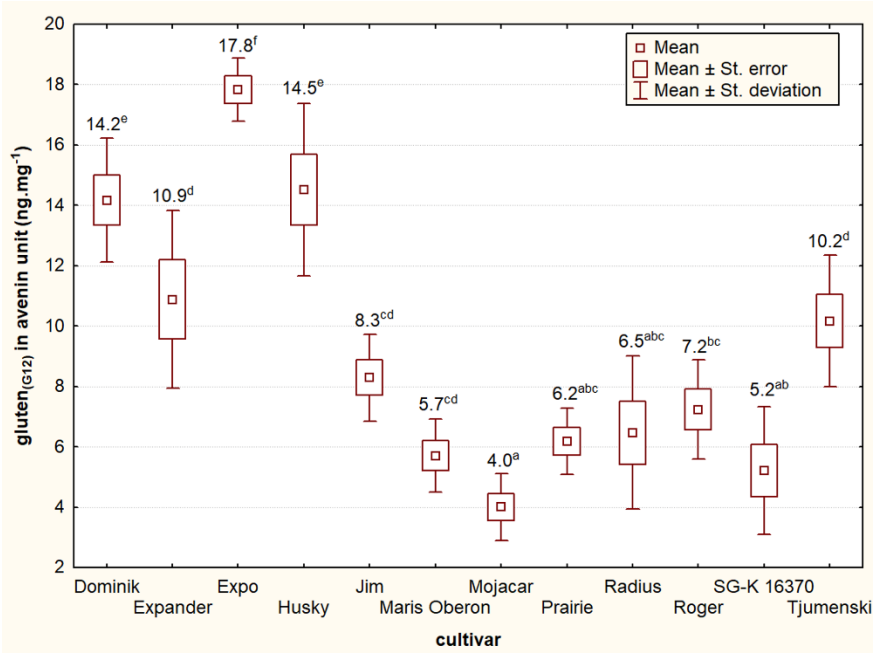

Fig. 5 Significant differences of gluten$_{(G12)}$ contents calculated on purified avenin unit in defined set of oat cultivars with the lower inter-year relative standard deviation (≤ 30 %) and the lowest average gluten$_{(G12)}$ content (≤ 5 mg . kg^{-1}) in dry matter of oat grain

*Cultivars marked with a different letter apostrophes are statistically significant at $p \leq 0.05$.

A comparison of electrophoretic variability of the bands around 30 kDa between these two groups with significantly different levels of gluten-like peptides revealed an occurrence of one unique band in genotypes with lowest gluten-like peptide content as well as an occurrence of two unique bands in genotypes with significantly higher gluten-like peptides content per avenin unit (Fig. 4A1 and A2). The differential and unique patterns in the position of electrophoretic bands around 30 kDa can represent perspective biochemical markers which has a promising potential of being utilized in oat breeding programmes aimed at selection of low gluten-like peptide content in avenin fraction.

The reasons underlying the discrepancies between the content of immunoreactive gluten-like peptides in grain dry matter and the analysis of purified avenin proteins can be interpreted in several ways. One possibility represents low reliability of predictions based on original kit measurements with regard to the values under the detection limit LOQ including the cross-reactivity of avenin-derived epitopes with wheat anti-gluten derived antibodies [6]. Significant differences in prediction of immunoreactive peptides levels depending on the tested matrix also have to be considered [24]. The increased levels of immunoreactive peptides could also be determined by further grain protein fractions such as non-precipitated gliadins and alcohol-soluble and insoluble glutelins. In bread wheat, three DQ2-restricted peptides were reported within glutelin fraction; however, their resulting levels are significantly lower in comaprison to gliadins considered as the major gluten allergen [25].

The detected significant content differentiations in gluten homologous epitopes based on purified avenin analysis indicates a higher potential of utilization of this approach for analyses of oat genotypes revealing low gluten-like reactivity. This approach thus represents a suitable method for identification of oat genotypes with low gluten reactivity as promising oat sources for CD patients.

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4. Conclusion

The obtained results confirmed a significant effect of both year (environment variation, seasonal variation) and genotype on the content of immunoreactive gluten-like peptides revealing a cross-reactivity with wheat anti-gliadin G12 monoclonal antibody in oat grains. With an exception of obvious contamination in French cultivar Sirene harvested in 2018, all other studied oat genotypes revealed immunoreactive gluten contents below the limit of 20 mg kg^{-1} in both years. However, only one third of the studied genotypes revealed relatively stable content of immunoreactive gluten-like peptides with RSD lower than 30% in both years of study. Detailed analysis of genotypes revealing relatively low gluten homological peptide content and relatively high inter-year stability led to an identification of 12 genotypes with significantly low gluten content around or below the detection threshold of the kit (LOQ 4 ppm). Purification of avenin fraction and the following analysis of avenin-derived gluten-like homologous led to the further differentiation within the group of oat genotypes with low gluten-like reactivity. Electrophoretic separation of purified avenins resulting in an identification of a unique band around 30 kDa which is specific for genotypes with very low gluten-like peptide content represents a promising biochemical marker for an identification of promising oat genotypes with low gluten-like peptide content in avenin fraction. Finally, the avenin purified procedure also revealed a discrepancy between the predicted gluten content based on this analysis and the content of gluten-like immunoreactive peptides based on grain analysis. It is evident that development of more accurate analytical approaches such as LC-MS based on the detection of gluten-like epitopes will be crucial for an identification of CD-safe oat materials.

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