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Immunogenicity characterization of hexaploid and tetraploid wheat varieties related to celiac disease and wheat allergy

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ABSTRACT

Wheat is one of the major cereals consumed throughout the world; there has been a radical increase in the population suffering from many wheat-related disorders. The present study was conducted to screen low immunogenic hexaploid and tetraploid wheat varieties. A total of 34 different wheat varieties were tested for its total protein content, gliadin content and immunoreactivity with immunoglobulins of celiac (IgA) and wheat allergy patients (IgE). The wheat varieties HD-2851 and NIAW-917 (hexaploid) and NIDW-295, UAS-428, PDW-291, MPO-1215 and DDK-1025 (tetraploid) were found to be less immunoreactive varieties for both celiac and wheat allergy patients. The immunoblot assays for IgE reactivity have revealed that gliadin subunits present in all varieties are found to be allergic proteins. The principal component analysis biplot showed that tetraploid wheat varieties are less immunoreactive than hexaploids. The low immunogenic wheat varieties are suitable for plant breeding and preparation of low gluten or hypo-immunogenic products.

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KEYWORDS

Wheat varieties; gliadin; celiac disease; wheat allergy; immunoreactivity

Introduction

Cereals and cereal-based products are indispensable in the day-to-day life of the majority of the world's population, and out of all the cereals consumed, wheat serves as an important source of the dietary proteins. Wheat belongs to the family of *Poaceae* under the genus *Triticum*. Globally, three major types of wheat are grown, hexaploid bread wheat (*Triticum aestivum*; AABBDD-genome; $2n = 42$ chromosomes), tetraploid (*Triticum durum*; AABB-genome; $2n = 28$ chromosomes) hard or durum wheat and diploid (*Triticum monococcum* AA-genome; $2n = 14$ chromosomes). Currently, 95% of the wheat grown globally is hexaploid and remaining 5% tetraploid wheat varieties. The hexaploid varieties like *T. aestivum* are utilized mainly in the preparation of bakery products, whereas tetraploid varieties like *T. durum* used in the preparation of pasta products represent most of the remaining 5% (Shewry, 2009). In a total of about 7–19.3% of proteins found in wheat, 80% is composed of gluten protein, which is made up of gliadin and glutenin subunits (GS)

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(Kucek, Veenstra, Amnuaycheewa, & Sorrells, 2015). Gluten proteins are rich in proline and glutamine amino acids and mainly contribute to the viscoelastic nature of dough, which gives rise to the preparation of a wide variety of wheat-based products (Battais, Richard, Jacquenet, Denery-Papini, & Moneret-Vautrin, 2008). Gluten is mainly responsible for the desirable strength and sensory properties of various products such as bread, cakes, flatbreads and pasta (Gallagher, Gormley, & Arendt, 2004).

Gluten proteins cause adverse effects on the health of certain individuals in spite of being a nutritionally important cereal for others. At present, populations afflicted with wheat-related disorders are increasing at a rate of around 0.5–2% worldwide (Rewers, 2005). Wheat-related disorders include wheat allergy, celiac disease (CD) and gluten intolerance. The wheat allergy includes allergy of skin and gastrointestinal tract, wheat-dependent exercise-induced anaphylaxis (WDEIA), occupational asthma (Baker's asthma) and rhinitis. The allergic reaction due to IgE antibodies produced against wheat proteins and peptides plays a significant role in the pathogenesis of these illnesses (Sapone et al., 2012). Previous reports have clearly indicated that the storage proteins of gliadin (α -, β -, γ - and ω -gliadin subunits) along with low molecular weight (LMW)-GS are the main allergy-inducing proteins in wheat (Battais et al., 2005; Lehto et al., 2003; Morita et al., 2003). CD is an autoimmune disorder with gluten-sensitive enteropathy causing an immune-mediated permanent intolerance to gluten. CD is a type of genetic autoimmune disorder, and it is caused mainly by switching the expression of HLA genes DQ2 or DQ8 in sensitive population. Wheat gluten protein is a main causing agent to stimulate the HLA genes to activate innate and adaptive immune response. Due to gastric digestion of wheat gluten proteins, it generates the immunogenic peptides, which damage the small intestine. When CD patients intake this gluten-containing foods, absorption of this gluten or gluten peptides stimulates the body immune system. It produces the autoantibodies against tissue transglutaminase (tTG) and gliadin-tTG complex, and these autoantibodies (anti-tTG) damage the intestinal villi structure (Bergamo et al., 2011; Berti et al., 2007; Lammers et al., 2008). The intestinal villi are usually involved in the absorption of nutrition. The damaged villi cannot absorb nutrition properly in the gut. So, CD patients must follow a strict gluten-free or low gluten diet throughout their lifetime. The recent survey reveals that 1% of the world population and about 1% of the North Indian community are suffering from CD. Another common type of wheat-related disorder, gluten intolerance, is mainly due to the indigestion of gluten proteins causing digestive problems such as gassiness, abdominal pain and diarrhea (Gallagher et al., 2004; Metakovsky, Knezevic, & Javornik, 1991).

During the last few centuries, many new wheat varieties have been released throughout the world. Although there are more than 20,000 wheat varieties around the world, the storage protein composition differs significantly due to their high polymorphic nature and increase in gluten content (Payne, 1987; Salentijn et al., 2013). Wheat breeding is a popular approach to improving the crop quality and yield. On the other side, it has been attracting researchers who are looking to improve the gluten proteins for its viscoelastic properties. According to Ribeiro et al. (2016), it has been suggested that breeding practices will not affect the toxic epitope peptides in the wheat varieties and the improvement of wheat varieties is based on the identification of genetic variants traits of interest in the wheat breeding practices. At the same time, it is possible that an increase in the consumption of fractionated wheat gluten used in the preparation of some food products might have resulted in the increased incidence of CD

(Kasarda, 2013). There is sufficient information available on the pathology, toxicology and allergic nature of different wheat varieties found across the globe. However, the existing literature lacks the segregation of less immunogenic wheat varieties for the Indian context, and it holds an important perspective for the selection of wheat for formulation and production of diversified wheat-based products for a wheat-sensitive population that has grown in the recent times.

The current study has been primarily focused on the screening of various wheat varieties from different parts of India for assessing their immunogenic behavior. Initially, the wheat varieties were analyzed for their total protein and gliadin contents. The immunogenicity of the wheat varieties was later examined using wheat allergen proteins and the sera of the wheat-sensitive patients, with IgE antibodies, for evaluation of wheat allergy, and with IgA antibodies for assessing the CD. The wheat allergen-triggering wheat proteins were characterized through immunoblot. This research study is expected to contribute mainly to better, improved and safer selection of wheat varieties for wheat breeding programs, and also for preparation of hypoallergenic or low gluten food products targeted for the wheat-sensitive population.

Materials and methods

Plant materials and chemicals

Thirty-four commercially available Indian wheat varieties were obtained from different parts of India. In these, 25 were *T. aestivum*, 6 were *T. durum* and 3 were *T. dicoccum*. The wheat varieties were obtained from the following places: Directorate of Wheat Research (Karnal), Mahatma Phule Krishi Vidyapeeth (Ahmednagar), Wheat Specialist (Niphad), Indian Agriculture Research Institute (New Delhi), Wheat & Maize Research Unit (Parbhani) and Agriculture University (Dharwad).

Antibodies like anti-gliadin–Horseradish peroxidase (HRP) conjugate, anti-Human IgE-ALP and anti-Human IgA HRP and gliadin were purchased from Sigma Aldrich, USA. All chemicals used for analysis were of analytical grade.

Collection of sera

Sera of wheat allergy patient's (donor patients) samples were obtained from the Allergy, Asthma and Chest Centre, Mysore. Sera of CD patients (donor patients) were obtained from AIIMS (All India Institute of Medical Sciences, New Delhi, India). Based on titer ELISA analysis with wheat proteins, 20 individual (CD patient) sera were selected and pooled according to the IgA titer levels against wheat flour proteins and 3 wheat allergy individual sera were pooled based on an IgE titer level. Control sera were obtained from normal individuals not having CD and allergic to wheat. All the sera samples were stored at -20°C for further analysis.

Milling of wheat varieties

All wheat samples were milled in laboratory scale mill (Laboratory Mill 3100, Perten Instruments, Sweden). Milled wheat flour samples were stored at 4°C for further analysis.

Total protein content

The total protein content of whole wheat flour was determined using total nitrogen content obtained by Kjeldahl method (American Association of Cereal Chemists [AACC], 2000).

Electrophoresis pattern of wheat flour proteins

Gliadin and glutenin proteins were extracted from wheat flour according to Van den Broeck et al. (2009). The protein extraction was carried out with 50% (v/v) aqueous isopropanol (1:10, w/v) and kept in continuous mixing at room temperature for 30 min. The extract solution was centrifuged at 10,000g for 15 min at room temperature. The residue was then extracted twice with 50% isopropanol in 50 mM Tris-HCl (pH 7.5) containing 1% dithiothreitol, for 30 min at 60°C. This was followed by centrifugation at 10,000g for 10 min. The protein content of all the extracts was quantified by Bradford assay and 10 µg of protein was loaded into 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method described by Laemmli (Bradford, 1976; Laemmli, 1970).

Characterization of gliadin subunits by immunoblot

Gliadin extraction was carried out as mentioned by Van den Broeck et al. (2009). Fifty milligrams of wheat flour sample was extracted with 1 ml of 60% aqueous ethanol for 1 h in continuous shaking (200 rpm). The supernatant was collected after centrifugation at 10,000g for 10 min. The residue was extracted twice with 60% ethanol, and all three extracts were pooled. The immunoblotting was carried out according to the method described by Lauriere (1993). Ten micrograms of gliadin extracts from different wheat varieties were separated on 12% SDS–PAGE. The separated proteins in the SDS–PAGE gel were transferred to the polyvinylidene difluoride (PVDF) membrane, and after the transfer, the membrane was kept in blocking solution containing 1% gelatin for 1 h. The blot was washed three times with phosphate buffer saline containing 0.05% Tween-20 (PBST) and followed by three 10 mM phosphate buffer saline (PBS) buffer washes. The blot was incubated in anti-gliadin-HRP conjugate solution for 2 h at 37°C, and then the blot was developed using an enhanced chemiluminescence substrate (Mickowska, Socha, & Urminska, 2016).

Quantification of gliadin using direct ELISA

The gliadin content in wheat varieties was quantified by using an RIDASCREEN Gliadin kit according to the method AOAC, 2012.01 (Immer & Haas-Lauterbach, 2012) (R7001, R-Biopharm, AG). Gliadin extraction and analysis were carried out according to manufacturer's instructions. The samples were further diluted in sample diluent buffer to fit within the standard range. The gliadin content was expressed in terms of percentage of flour.

Screening of low allergen wheat varieties using patient serum

Extraction of wheat proteins

The proteins from wheat flour samples were extracted with 5 ml of 4 M urea containing 0.1 M Tris-HCl buffer (pH 8.6) by adding 40 mg sample each. Extraction was carried out by

overnight shaking at 4°C. The extract supernatant was collected by centrifugation at 5000 rpm for 10 min. The extracts were kept at 4°C for further immunoreactivity assay with CD and wheat allergic patient's sera (Nakamura, Tanabe, Watanabe, & Makino, 2005).

Immunoreactivity of wheat varieties to IgA antibodies of CD patient's sera

The extracted wheat variety proteins were coated in 1:1000 dilutions (0.2 M bicarbonate buffer pH 9.6, 100 µl/well) into each well and incubated overnight at 4°C. After blocking with 1% gelatin for 1 h, 100 µl of 1:300 diluted sera was added to the wells and incubated at RT for 2 h 30 min. The wells were washed thrice with 10 mM PBST and followed by three 10 mM PBS buffer washes. Hundred microliters of anti-human IgA-ALP (alkaline phosphatase) conjugate (1:35,000 dilution) was added to each well. The wells were washed with 10 mM PBST after 2 h incubation, followed by three 10 mM PBS buffer washes. Hundred microliters of the pNPP (*p*-Nitrophenyl phosphate) substrate was added to each well. After incubating for 30 min under dark, the reaction was stopped by adding 50 µl of 3 N NaOH. The pooled sera from three healthy individuals were used as negative control. The results are expressed in terms of relative ELISA value (Leszczynska et al., 2013; Vainio, Kalimo, Reunala, Viander, & Palosuo, 1983).

Immunoreactivity of wheat varieties to IgE antibodies of wheat allergy patient's sera

IgE ELISA was carried out as described by Nakamura et al. (2005). The extracted wheat proteins in the 4 M urea buffer (pH 8.6) samples were diluted in 0.2 M bicarbonate buffer pH 9.6 (1:1000 dilutions), and 100 µl was added to the wells. The plate was incubated at 4°C overnight. After blocking with 1% gelatin for 1 h, 100 µl of 1:5 diluted sera was added to the wells and incubated at RT for 2 h and 30 min. The wells were washed thrice with 10 mM PBST and followed by three 10 mM PBS buffer washes. Hundred microliters of the anti-human IgE-ALP conjugate (1:6000) was added to each well. After 2 h of incubation, the wells were washed thrice with 10 mM PBST/PBS, and 100 µl of the pNPP substrate was added to each well. After incubating for 30 min under dark, the reaction was stopped by adding 50 µl of 3 N NaOH. The pooled sera from three healthy individuals were used as negative control. The results were expressed in terms of relative ELISA value.

Immunoblot: IgE and IgA immunoreactivity with wheat proteins

Immunoblot was carried out to identify the IgE and IgA binding wheat proteins. The extracted proteins from different wheat varieties were separated in 12% SDS-PAGE (reducing). The separated protein gels were transferred to PVDF membrane, and the membrane was kept in blocking solution containing 1% gelatin for 1 h. The blots were washed thrice with 10 mM PBST and followed by PBS buffer. The blots were incubated with the sera of wheat allergic patients' (1:25 dilution) and CD patients' sera (1:100) at 37°C for 3 h. The respective blots were incubated with anti-Human IgE-ALP conjugate and anti-Human IgA-ALP conjugate incubated at 37°C for 2 h. The blots were washed with 10 mM PBST and followed by PBS wash, and the reaction was developed by adding BCIP/NBT

(5-bromo-4-chloro-3-indolyl-phosphate-nitro blue tetrazolium) and incubated for 30 min at 37°C (Klockenbring, Boese, Bauer, & Goerlich, 2001).

Statistical analysis

All the experiments were performed in triplicates, and the data were expressed as mean $\pm$ standard deviation. Principal component analysis (PCA) was performed using XLSTAT, version 2008.04.02.

Results

Total protein content, SDS-PAGE profile of wheat proteins and gliadin subunits

The total protein content of wheat varieties is shown in Table 1. The highest amount of protein content was observed in DDK-1009 (15.89%), whereas it was found to be lowest in “SUJATA” (9.00%). Gluten proteins were extracted from 34 wheat varieties

Table 1. Total protein and gliadin contents of different hexaploid and tetraploid wheat varieties.

Sl. No	Species	Variety	Total protein content (%)	Gliadin (%)
1	<i>T. aestivum</i>	NIAW-301	11.51 $\pm$ 0.08	5.41 $\pm$ 0.01
2		WR-544	13.11 $\pm$ 0.16	3.87 $\pm$ 0.15
3		SIWOR	10.37 $\pm$ 0.01	5.39 $\pm$ 0.03
4		HD-2851	11.97 $\pm$ 0.01	4.07 $\pm$ 0.14
5		NIAW-1885	11.40 $\pm$ 0.08	4.92 $\pm$ 0.12
6		DBW-71	12.42 $\pm$ 0.01	3.99 $\pm$ 0.36
7		NIAW-34	11.17 $\pm$ 0.01	4.85 $\pm$ 0.23
8		MP-147	10.83 $\pm$ 0.00	4.64 $\pm$ 0.27
9		PBN-51	14.25 $\pm$ 0.16	5.47 $\pm$ 0.37
10		AJANTA	14.70 $\pm$ 0.01	4.36 $\pm$ 0.10
11		HD-2687	11.97 $\pm$ 0.01	6.71 $\pm$ 0.09
12		NIAW-1415	11.97 $\pm$ 0.01	5.58 $\pm$ 0.12
13		NIAW-1994	10.60 $\pm$ 0.08	4.44 $\pm$ 0.10
14		SUJATA	9.00 $\pm$ 0.01	5.70 $\pm$ 0.18
15		MP-CROSS	10.60 $\pm$ 0.16	3.85 $\pm$ 0.13
16		NIAW-917	12.31 $\pm$ 0.01	4.23 $\pm$ 0.15
17		DWR-162	12.76 $\pm$ 0.16	3.87 $\pm$ 0.18
18		UAS-304	14.13 $\pm$ 0.16	4.54 $\pm$ 0.02
19		LOK-1	11.74 $\pm$ 0.01	5.05 $\pm$ 0.23
20		PBW-502	9.46 $\pm$ 0.01	3.81 $\pm$ 0.12
21		SONALIKA	10.26 $\pm$ 0.01	5.57 $\pm$ 0.23
22		MP-4010	12.88 $\pm$ 1.45	5.41 $\pm$ 0.32
23		C-306	11.97 $\pm$ 0.01	3.20 $\pm$ 0.36
24		MACS-6222	10.71 $\pm$ 0.01	4.31 $\pm$ 0.30
25		DBW-17	12.42 $\pm$ 0.01	5.55 $\pm$ 0.21
26	<i>T. durum</i>	NIAW-15	14.59 $\pm$ 0.08	4.21 $\pm$ 0.15
27		PDW-291	11.97 $\pm$ 0.01	3.39 $\pm$ 0.23
28		NIDW-295	11.28 $\pm$ 0.16	2.03 $\pm$ 0.08
29		MPO-1215	11.62 $\pm$ 0.40	3.38 $\pm$ 0.18
30		UAS-415	13.22 $\pm$ 0.16	3.17 $\pm$ 0.17
31		UAS-428	13.16 $\pm$ 0.08	3.43 $\pm$ 0.05
32	<i>T. dicoccum</i>	DDK-1025	13.68 $\pm$ 0.01	3.39 $\pm$ 0.07
33		DDK-1029	13.68 $\pm$ 0.16	4.33 $\pm$ 0.28
34		DDK-1009	15.89 $\pm$ 0.03	5.61 $\pm$ 0.19

Note: The results are expressed in terms mean $\pm$ SD. Experiment was carried out in triplicate.

and separated by SDS–PAGE. The protein pattern in the SDS–PAGE indicates that the high molecular weight (HMW)-GS proteins were found in the range of 80–100 kDa, D-type LMW-GS and ω -gliadin were found in the range of 45–60 kDa and α -, β - and γ -gliadins and B/C type LMW-GS were found at 30–45 kDa region. The *T. aestivum* species can be easily differentiated from other species by observing the HMW-GS, and significant differences were observed in HMW-GS among hexaploid and tetraploid varieties. The *T. aestivum* varieties have three to four HMW-GS, whereas tetraploid (*T. durum* and *T. dicoccum*) species have one to two prominent HMW-GS. Also, UAS-415 and UAS-428 (*T. durum*) varieties display similar protein profiles. The proteins banding pattern of different wheat varieties is shown in Figure 1. Immunoblotting was performed to identify gliadin subunits pattern among the wheat varieties. Gliadin subunits were found in 30–45 kDa region. Results indicated that 6–8 gliadin subunits were found in wheat varieties, namely, SONALIKA, NIAW-34, UAS-304, NIAW-1994, SUJATA, WR-544, HD-2851, C-306, NIAW-301, DWR-162 NIAW-1885, MPO-1215, UAS-415, UAS-428, MP-CROSS, NIDW-295 and PDW-291. The gliadin protein profile of different wheat varieties is shown in Figure 2.

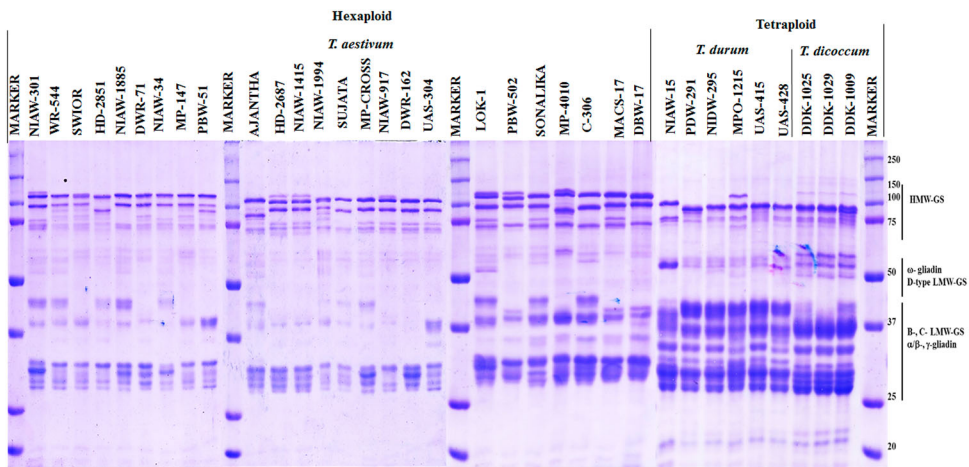


Figure 1. Separation of gliadin and GS from different wheat varieties by SDS–PAGE.

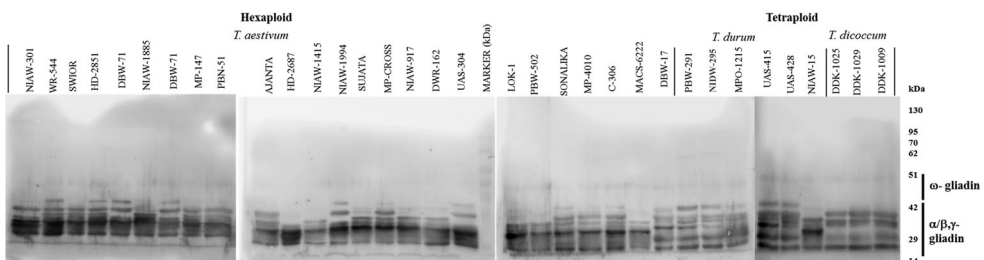


Figure 2. Reactivity in immunoblotting of anti-gliadin antibodies with the extracted gliadins from different wheat varieties.

Gliadin contents in wheat varieties

The gliadin content was analyzed among the hexaploid and tetraploid wheat varieties in which high gliadin content (Table 1) was observed in *T. aestivum* varieties, namely, HD-2687 (6.71%), followed by SUJATA (5.70%), DBW-17 (5.55%) PBN-51 (5.47%), NIAW-301 (5.41%) and SIWOR (5.39%). In the case of *T. durum* and *T. dicoccum*, gliadin content was higher in the variety DDK-1009 (5.61%). Low gliadin content was observed in C-306 (3.20%) followed by PBW-502 (3.81%), WR-544 (3.87%) and DWR-162 (3.87%). Among tetraploid varieties, NIDW-295 (2.03%), UAS-415 (3.17%), UAS-428 (3.43%), MPO-1215 (3.38%) and DDK-1025 (3.39%) showed lower gliadin content. The gliadin content varied among the wheat varieties is shown in Table 1.

Reactivity of IgA antibodies (CD patients) with wheat proteins

Immunoreactivity of wheat varieties with IgA antibodies was observed in the range of 0.112–0.695 relative ELISA value among which UAS-428 (0.112), NIDW-295 (0.126), PDW-291 (0.171), HD-2851 (0.189), MPO-1215 (0.227) MP-CROSS (0.247), SUJATA (0.248), SIWOR (0.273) and MP-147 (0.281) were less immuno-reactive depending on the IgA relative ELISA value. The tetraploid variety UAS-428 (0.112) was found with 6.2-fold less immunoreactivity as compared to the hexaploid varieties DWR-162 (0.695). The wheat varieties DBW-71 (4.6), USA-304 (4.4) and NIAW-301 (4.1) have 4-fold more immunoreactive than UAS-428. IgA antibodies of CD patients showed a significant difference in the immunoreactivity between hexaploid and tetraploid wheat varieties. The immunoreactivity of wheat varieties is shown in Figure 3.

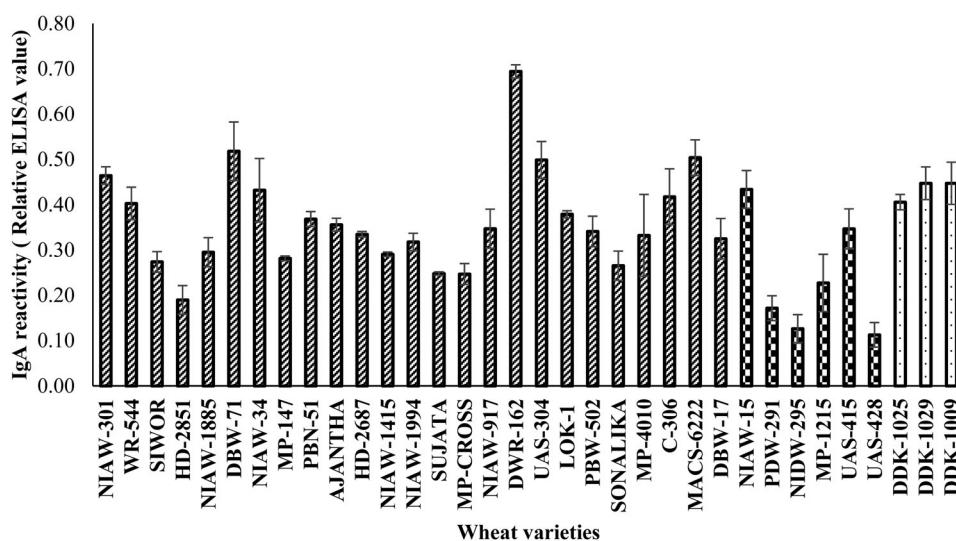


Figure 3. Reactivity of CD patients' serum IgA with different wheat varieties. The results are expressed in terms of relative ELISA value mean $\pm$ SD. *T. dicoccum* (▨), *T. durum* (■) and *T. dicoccum* (□).

Reactivity of the IgE antibodies (wheat allergy patients) with wheat proteins

The protein extracts of 34 wheat varieties showed different immunoreactivity with the IgE of patient serum samples. A significant difference was observed in the relative ELISA value among these wheat varieties, which was found to range from 0.24 to 1.07. Among all hexaploid wheat varieties, NIAW-917 (0.320), WR-544 (0.342), PDW-291 (0.361) and HD-2687 (0.364) were found to be less immunoreactive, and in case of tetraploid wheat varieties, NIDW-295 (0.247), MPO-1215 (0.280), UAS-415 (0.304), UAS-428 (0.306), NIAW-15 (0.361), DDK-1009 (0.291), DDK-1025 (0.422) and DDK-1029 (0.469) showed less reactivity. The highest IgE reactivity was seen in wheat varieties such as SONA-LIKA (0.805), MP-147 (0.877), SUJATA (0.892), C-306 (0.900), NIAW-1885 (0.931), NIAW-1885 (0.931), NIAW-301 (0.962), LOK-1 (1.016) and DBW-71 (1.071). The wheat variety NIDW-295 showed a low IgE relative to the ELISA value of 0.246, when we compared with other wheat varieties NIAW-301 (0.962), LOK-1(1.016) and DBW-71 (1.071), these wheat varieties having 3.9, 4.1 and 4.3 fold more immunoreactivity. The IgE reactivity with protein extracts of different wheat varieties is shown in Figure 4.

Immunoblot: IgE and IgA reactivity with wheat proteins

The extracted wheat protein samples were subjected to immunoblot using IgE antibodies (Figure 5(a)) to characterize the IgE binding protein subunits. A high degree homology of the IgE binding pattern was observed in 30–60 kDa region, and less immunoreactivity to HMW-GS protein bands was observed. The IgE antibodies were seen to be reacting more prominently with α -, β - and γ -gliadin subunits, which indicates that these subunits are more immunoreactive to wheat allergic patients. The reactivity of ω -gliadin bands was found in the following wheat varieties, namely, UAS-425, UAS-428, SUJATA, NIAW-1885, PDW-291, MPO-1215, NIAW-1994, C-306 and NIAW-301, whereas other varieties

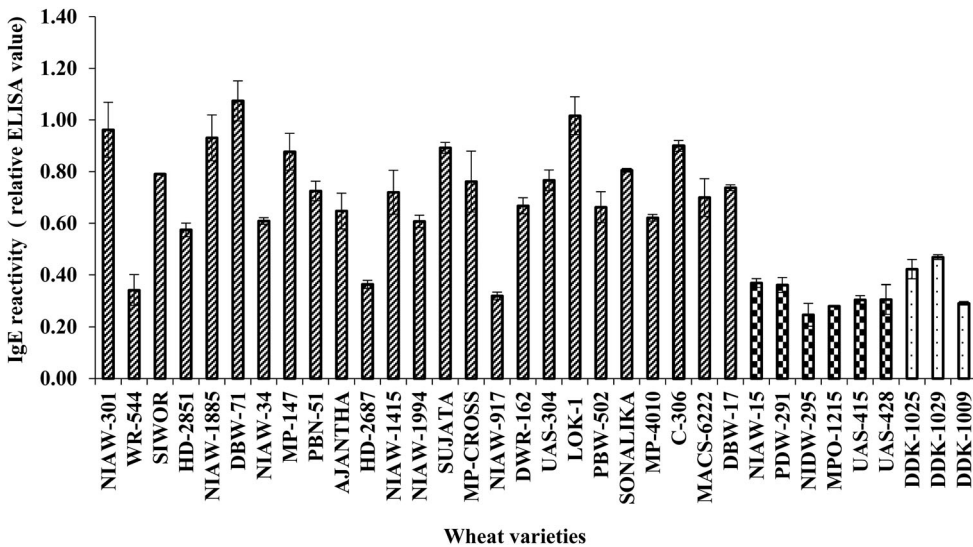


Figure 4. IgE reactivity with different wheat. The results are expressed in terms of relative ELISA value mean $\pm$ SD. *T. dicoccum* (▨), *T. durum* (■) and *T. dicoccum* (□).

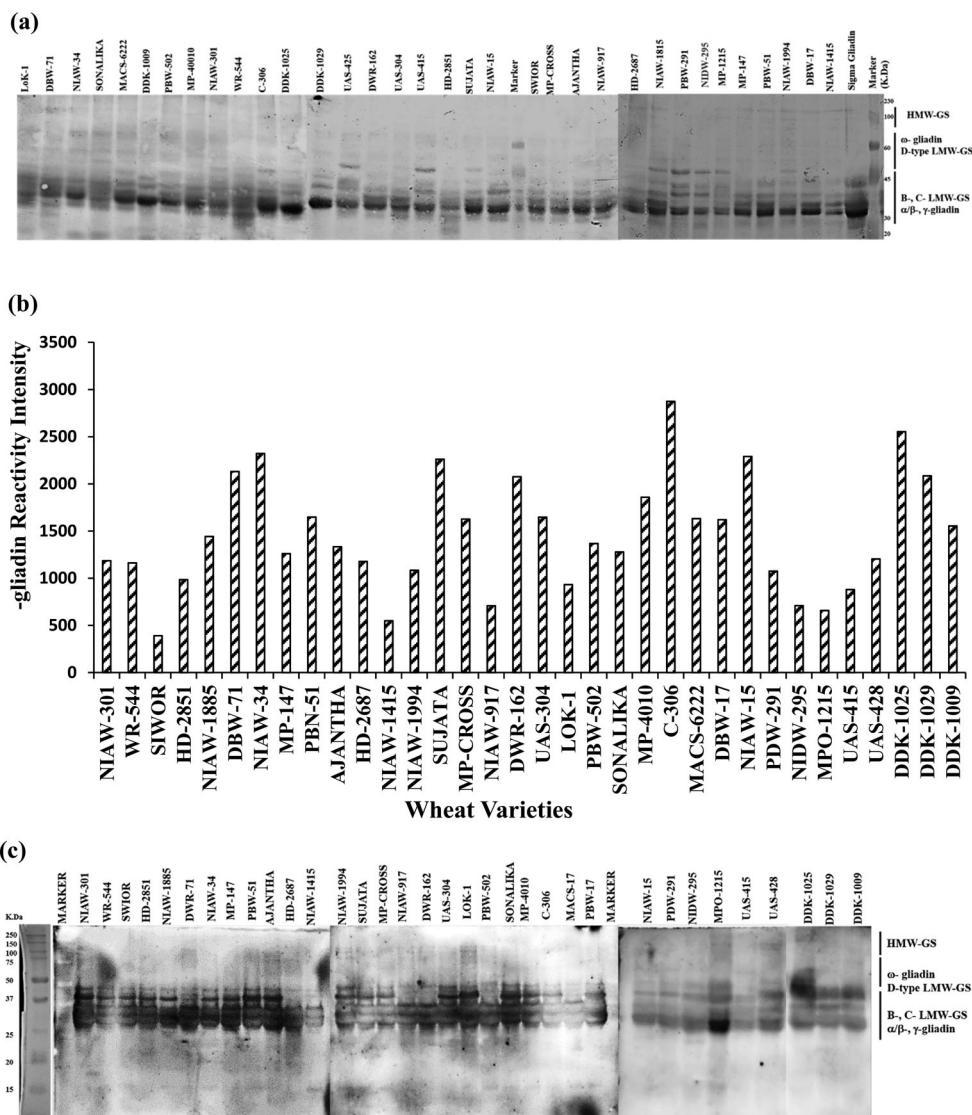


Figure 5. Immunoblot with patient's sera. (a) Wheat allergic patient's IgE immunoreactivity with wheat proteins, (b) α -gliadin band reactivity intensity with IgE antibodies, (c) CD patient IgA reactivity with wheat proteins.

exhibited very less intense bands. The IgE immunoblot α -gliadin was quantified by ImageJ software. The IgE antibodies were seen to be reacting more prominently with α -, β -, and γ -gliadin subunits. In these, α -gliadin subunit found is more reactive subunit in the all the varieties, so further α -gliadin band intensity was measured to check the reactivity among the varieties in which C-306, DDK-1025, DDK-1029, NIAW-34 and DBW-71 varieties showed a high intensity, and HD-2851, NIDW-295, MP-1215, NIAW-1415, SIWOR, NIAW-917 and MP-cross varieties had less band intensity (Figure 5(b)). Immunoblot analysis of wheat proteins that explored CD patients' IgA antibodies (Figure 5(c)) showed a strong reactivity profile with wheat varieties proteins. IgA reactive pattern

was found to be strong in the gliadin subunits region in all wheat varieties. It indicates that gliadin subunits are more immunoreactive due to the presence of immunogenic epitopes compared to other wheat glutenins.

Principal component analysis

PCA is a commonly adopted statistical tool used for interpreting and visualizing groupings of samples and recognizing new meaningful underlying variables. The study has provided insight into the distribution of wheat varieties based on their immunoreactivity. PCA was carried out based on the gliadin content, IgA and IgE reactivity (Figure 6). Gliadin content falls in the first component, IgE and in the secondary component, IgA. The principal component or factors retained 51.06% and 28.93% of the initial sample variability, respectively, and explained 79.99% variation of the samples considered. It has been observed that there is particular cluster formation in wheat varieties (*T. aestivum*, *T. durum*, and *T. dicoccum*) based IgE, gliadin and IgA reactivity. The wheat varieties in quadrant IV SUJATA, HD-2687, SONALIKA, SIWOR, NIAW-1415, NIAW-1885, DBW-17, PBW-51, MP-4010 and LOK-1 are found close to the gliadin and IgE reactivity. The gliadin content and IgE reactivity are located in the same quadrant which implies a strong correlation between these two components, and it can be observed that the varieties having high gliadin contents have high IgE reactivity. Wheat varieties DWR-162, C-306, MACS-6222, UAS-304, DBW-71 and NIAW-301 are found close to the IgA reactivity. Wheat varieties DDK-1025, DDK-1029, C-306, MACS-6222, UAS-304, DBW-71 and NIAW-301 are found close to the IgA reactivity. Wheat varieties DDK-1025, DDK-1029, C-306, MACS-6222, UAS-304, DBW-71 and NIAW-301 are found close to the IgA reactivity.

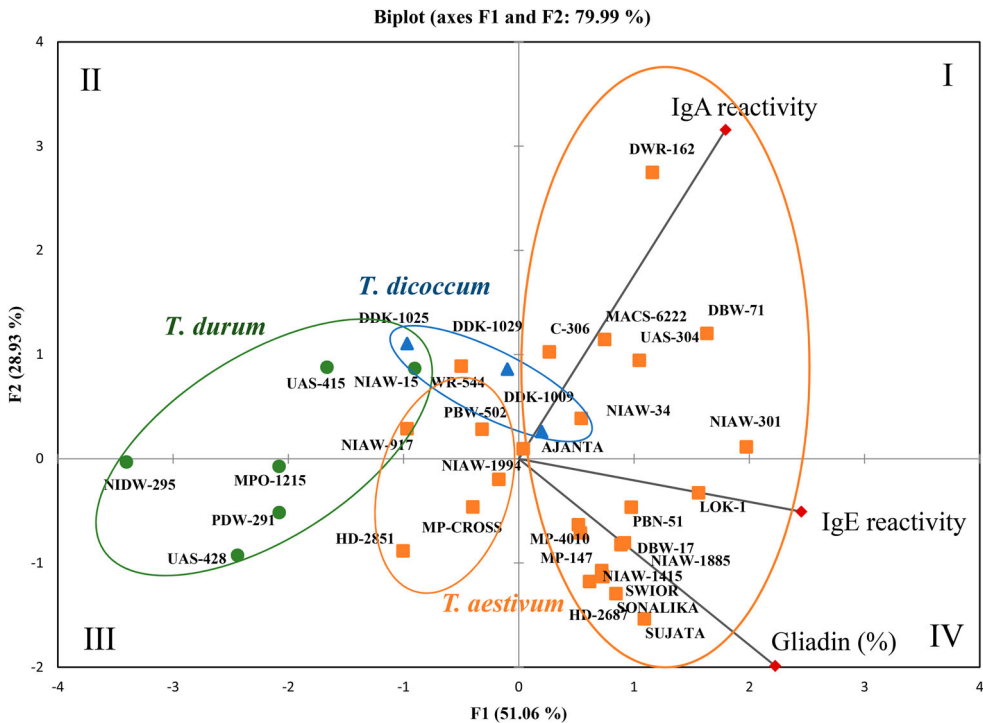


Figure 6. PCA of gliadin, IgA IgE reactivity with wheat varieties. Variables gliadin content, IgA and IgE reactivity. Gliadin content made as a first component, IgE; IgA as secondary components. Wheat varieties *T. aestivum* (■), *T. durum* (●) and *T. dicoccum* (▲).

6222, DBW-71, UAS-304, NIAW-34, DDK-1009, AJANTA and NIAW-301 present in quadrant-I along with IgA reactivity represent that they are closely related and have more IgA reactivity. These wheat varieties have more IgA reactivity compared to the other quadrants. The wheat varieties UAS-415, DDK-1025, WR-544, NIAW-917, NIAW-15, MP-CROSS, DDK-1029 and PBW-502 are found in quadrant-II, which is opposite to the quadrant comparing gliadin content and IgE reactivity, inferring that they are indirectly related and showed low values of these components. The wheat varieties NIDW-295, UAS-428, PDW-291, MPO-1215, HD-2851, MP-CROSS and NIAW-1994 are present in quadrant-III and these wheat varieties are away from gliadin content as well as IgA and IgE reactivities. Wheat varieties in quadrant-II and -III were found to be less immunogenic than wheat varieties in quadrant-I and -IV. The data visualization by PCA revealed that tetraploid (*T. durum*) wheat varieties are found in the quadrant-II and -III and are related to each other. The hexaploid wheat varieties are maximum placed in the quadrant-I and -IV, implicating that the hexaploid varieties are comparatively more immunoreactive than the tetraploid wheat varieties. PCA plot is shown in Figure 6.

Discussion

An upward growth in the consumption of wheat products around the globe is observed for the last five decades. Wheat-based products play an indispensable role in the day-to-day life in the form of bread, pizza, cakes, Indian typical flatbreads and other bakery products. In recent years, there are many wheat varieties in the market with high yield and improved bread-making quality. Although these new varieties have high gluten content to improve the product quality and are high on market demand, there is a steady increase in the population suffering from wheat-related disorders lately. Presently, many researchers are being focused on comparative genetic analysis at protein expression level and toxic peptides among wheat varieties. Wheat proteins are difficult to digest, and the absorption of gluten by the body is hindered either at the beginning of childhood or adult stage or by way of genetic disease.

Gliadin is a major immunogenic food protein for both CD and wheat allergy. Previous literature has already revealed that the repetitive sequences of gliadin play a major role in the immunogenicity and α -, β -, γ - and ω -gliadins are the major activators of the CD (Kucek et al., 2015). Wheat proteins differ in their stimulation response in allergic patients. In terms of stimulation, α/β -gliadins and LMW-GS show 60%, γ -gliadins show 55%, ω -gliadin shows 48% and HMW-GS stimulate 26% (Battais et al., 2003). The gliadin quantification and immunoblot results confirmed that the gliadin content differed among varieties which may be due to genetic variation and environmental factors.

The immunoblot confirms that α -, β - and γ -gliadins and LMW-GS were found to be more immunoreactive with IgE and IgA antibodies of patients' sera. A similar result is indicated by Bouchez-Mahiout et al. (2010) that gliadins and LMW-GS of wheat extract show IgE reactivity with patients' sera. *T. aestivum* (LOK-1, SIWOR, MP-147 and HD-2851) and *T. durum* (NIAW-15, UAS-415, NIDW-295, UAS-428 and PDW-291) showed less reactivity with the sera of CD patients, which indicated that the maximum tetraploid varieties are less immunoreactive when compared to the hexaploid varieties. It has been identified that tetraploid varieties showed the least amount of T-cell stimulatory peptides by using the

Glia- α 9 and Glia- α 20 antibodies (van den Broeck et al., 2010). These are because the hexaploid varieties have more immunoreactive epitopes compared to tetraploid varieties due to the presence of the D-genome in hexaploids. These D-genomes are highly expressive immunogenic gliadins in hexaploid varieties (Molberg et al., 2005; Van Herpen et al., 2006). It has also reported that tetraploid wheat varieties such as *Emmer* and *durum* are less immunoreactive than common bread wheat varieties (Auricchio, De Ritis, De Vincenzi, Occorsio, & Silano, 1982). Our results are similar to those of Leszczynska et al. (2013) who have stated that tetraploid species of *T. durum* and *Triticum aethiopicum* had low immunoreactivities towards peptide antibodies. Four varieties from *T. aestivum*, five from *T. durum* and one from *T. dicoccum* were found to be less allergenic. The highest relative ELISA value of 1.1 was observed in DBW-71 (hexaploid) variety, and the lowest value was observed in NIDW-295 (0.24) (tetraploid), which is about 4.5-fold less immunoreactive. Nakamura et al. (2005) also screened the less allergenic wheat varieties using allergic patient sera and concluded that *Einkorn*, *Rivet* and other eight varieties were found to be least allergic (Nakamura et al., 2005). Many researchers also reported that α/β -gliadin is more allergic due to more repetitive sequences of proline and glutamine amino acids. Less allergic wheat varieties having low T-cell stimulatory epitopes can be highly useful in the preparation of modified gluten products and development of less allergic varieties through breeding. PCA reveals that tetraploid wheat varieties (*T. durum*, *T. dicoccum*) were found less immunogenic than the hexaploid wheat varieties. The reason is the presence of D-genome in hexaploid (AABBDD-genome), whereas in tetraploid, it is absent (AABB-genome) (Kasarda, 2013). The PCA revealed that tetraploid (*T. durum* and *T. dicoccum*) wheat varieties are found in the quadrant-II and -III and are related each other. The biplot gives a clear visualization of wheat varieties distribution depending on the immunoreactivity. The hexaploid wheat varieties are maximum placed in the quadrant-I and -IV, implicating that the hexaploid varieties are immunoreactive compared to the tetraploid wheat varieties. The low allergic or less immunoreactive wheat varieties can be used for the enzymatic or microbial modification for the preparation of hypo-immunogenic food products targeting the wheat-sensitive population. Many researchers used the protease enzyme to modify the wheat gluten protein and reduce its immunogenicity followed by preparation of hypoallergenic pasta (Susanna & Prabhasankar, 2015). The Leszczynska et al. (2009) also modified the wheat flour to decrease the immunoreactive gliadin proteins using the lactic acid bacteria.

Conclusion

The aim of our study is to conduct and make contribution to the development of immunoreactivity of the hexaploid and tetraploid wheat varieties with the sera of CD and wheat allergy patients. Due to increase in population with wheat-related disorder all over the world, less immunogenic wheat varieties are in high demand. The results revealed that tetraploid wheat varieties were comparatively less immunogenic than hexaploid varieties. Immunoblot confirms that gliadin subunits are significant allergic proteins among the screened varieties. From this study, we conclude that among the hexaploid, HD-2851 and NIAW-917 varieties and among the tetraploid, NIDW-295, UAS-428, PDW-291, MPO-1215 and DDK-1025 wheat varieties were found to be less immunoreactive. The selective low allergic or less immunoreactive wheat varieties can be utilized for plant

breeding, and for the formulation, modification (enzymatic or microbial) and preparation of hypo-immunogenic food products targeting the wheat-sensitive population. This study paves the way for boosting the development and consumption of less immunogenic wheat varieties to reduce the risk factors for the future generation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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