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Understanding the Effects of Genotype, Growing Year and Breeding on Tunisian Durum Wheat Allergenicity (Part 2): The celiac disease case

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Abstract:

The aim of this study was to compare immunogenic and toxic gluten peptides related to celiac disease (CD). 100 accessions of genotypes selected during the 20th century in Tunisia were *in vitro* digested, then analyzed by UPLC/ESI-MS technique using an isotopically labeled internal standard. The first MANOVA confirmed a high variability in the content of immunogenic and toxic peptides reflecting high genetic diversity in the germplasm released during the last century in Tunisia, consistently with PCA and clustering analysis results. Our finding showed also important variability in CD epitopes due to growing season's climate scenarios. Moreover, the second MANOVA revealed significant differences between abandoned and modern cultivars CD-related peptide amounts. Although we could not conclude that there was an augment of allergens in newly selected durum wheat lines compared to abandoned ones, we demonstrated that modern genotypes peptides were less sensitive to climate variation, which is a useful indicator for wheat breeders.

Key words: Celiac disease, immunogenic, toxic, gluten, breeding, MANOVA

Introduction

Celiac disease (CD) is known as gluten-sensitive enteropathy and estimated to affect approximately 1–2% of the populations of North and South America, India, Europe and the Indian subcontinent [1, 2, 3]. However, in North Africa the diagnostic rate is still very low, mostly due to low availability of diagnostic facilities and poor disease awareness [4.] CD may be defined also as an inflammatory disease of the upper small intestine (duodenum, jejunum) in genetically susceptible individuals caused by the ingestion of gluten proteins from wheat, rye, barley, and possibly oats [5, 6]. The toxic protein fractions of gluten include gliadins and glutenins, with gliadins containing monomeric proteins and glutenins containing aggregated proteins [7]. Gluten subunits display molecular weights between 20 and 75 kDa, and contain similar or repetitive glutamine and proline rich peptide sequences [8]. Gliadins are supposed to be the active fractions of gluten; in fact, they contain the immunogenic peptides (especially the 33mer) and are able to exert a direct cytotoxic effect on the cell [9]. LMW-glutenin proteins presented also several motifs associated with the induction of CD [10].

Interplay between innate and adaptive immune responses to ingested gluten is involved in CD [11]. Gluten could have a direct toxic (innate) effect on the intestinal epithelial cells (IECs); while the adaptive immune response involves $CD4^+$ T cells in the lamina propria that recognize processed gluten epitopes [12,13]. The immune response against prolamins of toxic cereals is mediated through cytokines produced via both innate and adaptive immune branches [14]. Therefore, gluten epitopes might be subdivided into type's fragments; toxic peptides are able to induce mucosal damage, whereas immunogenic peptides are able to specifically stimulate HLA-DQ2- or DQ8- restricted T cell lines and T cell clones[13]. Thus, the development of methods for quantitative determination is important to understand the exposure thresholds and to support clinical allergy study designs [15]. Mass spectrometry (MS) is now established as an important tool for analyzing proteins and their proteolytic degradation mixtures of peptides, mainly because of its high sensitivity, speed, and to small sample-size requirement [16]. Because the reasons behind the increased prevalence of CD in the last 50 years are not fully understood, using such a highly sensitive and novel diagnostic tool is fundamental [17].

CD pathogenesis is the result of the interaction of a series of complex mechanisms involving genetic, immunological and the most likely environmental, such as a change in quantity, quality, or processing of cereal [18, 19]. To date, gluten is the only known environmental factor

to play a direct causal role in CD, and the only treatment for CD is a gluten-free diet (GFD) [20]. It has been speculated that the increase in CD may have occurred because of changes in wheat proteins that resulted from wheat breeding (mainly an increase in the gluten content, which is directly proportional to protein content) [21]. In this regard, the question if the history of wheat breeding from elite to modern lines promoted immunogenicity, has long existed. Such a subject matter could put forward the magnitude of the comparisons between landraces and old genotypes (with minor genetic modification) and modern genotypes (with major genetic modification). Therefore, CD would be an ideal model to study the natural history of autoimmune diseases [22].

A few studies have focused on the impact of breeding programs on the dark side of wheat. Breeding versus wheat pathogenicity could be exposed through the screening of wheat historical series epitopes related to CD. In fact, in this research paper, the trait of concern is to determine breeding history influence on wheat immunogenic and toxic potential of Tunisian durum wheat (landraces, old and modern cultivars). To consider the effect of genotype, growing seasons and breeding programs on the content of peptides associated to CD, the panel of peptides that survived in *vitro* static digestion was characterized by liquid chromatography- mass spectrometry (UPLC/ESI-MS) and then statistically analyzed.

Material and methods

Plant material

Tunisian durum wheat historical series were grown during three seasons (2011-2012, 2013-2014 and 2014-2015) in the trial field of the Graduate School of Agriculture of Kef, Tunisia:

Set 1 (season 2014-2015) comprises 70 accessions of durum wheat consisting in landraces, old cultivars, advanced genotypes made by international breeding programs (CYMMIT and ICARDA) and recent lines selected by national breeding program (INRAT). The set was subdivided into 2 groups according to their actual situation:

- Group 1 made up of 59 abandoned lined released from 1900 to 1979: 51 indigenous and exotic landraces (selected from 1900 to 1940) (Hmira (3 accessions), Jenah Khotifa (3 accessions), Azizi (3 accessions), Aouij (1 accession), Sbei (2 accessions), Derbassi (2 accessions), Ward lebled (2 accessions), Biadh (2 accesions), Swabaa elgia (2 accessions), Chetla (2 accessions), Roumani (2 accessions), Kmiret Zarzis (1 accession), Aoudy (2 accessions), Richi (2 accessions), Biskri (3 accessions), Agili (3

accessions), Arbi (2 accessions), Mahmoudi (3 accesions), Bidi (3 accessions), Bidi 17 (1 accession) and GT (7 accessions)), 7 old cultivars (from 1940 to 1970) ((Chili (3 accessions), Kyperounda (1 accession)), (INRAT 69 (2 accessions) and Badri (1 accession)) and 1 CIMMYT genotype (from 1970 to 1980) Maghrebi (1 accession)

- Group 2 comprised 11 modern genotypes (from1979-recent years): CIMMYT genotypes (Karim (1 accession) and Khiar (2 accessions)), ICARDA genotypes ((Waha (2 accessions), Om rabiaa (2 accessions) and Nasr (2 accessions)) and INRAT (Maali (2 accessions)).

Set 2 (season 2013-2014) comprised 15 accessions:

- Group 1: 6 abandoned lines (landraces (Hamira, Jenah Khotifa, Richi, Beskri, Mahmoudi and Bidi) and 3 old cultivars (Chili, Kyperounda and INRAT 69))
- Group 2: 6 modern genotypes (2 CIMMYT (Karim and Khiar),2 ICARDA (Om Rabia and Nasr) and 2 INRAT (Maali and Salim))

Set 3 (2011-2012) comprised 15 accessions:

- Group 1 consisted of 6 landraces (Hamira, Jenah Khotifa, Richi, Beskri, Mahmoudi and Bidi) and 3 old cultivars (Chili, Kyperounda and INRAT 69)
- Group 2 formed by 6 high yielding varieties; 2 made in CIMMYT (Karim and Khiar), 2 in ICARDA (Om Rabia and Nasr) and 2 in INRAT (Maali and Salim).

Experimental setup

The experimental trials of the three seasons of cultivation were conducted in the trial field of the Graduate School of Agriculture of Kef (Tunisia). This field had a clay-loam soil with mean values of 48% clay, 30% loam, 21.25 % sand, 184 ppm total nitrogen (Kjeldahl method), 3.92 ppm assimilable phosphorus (Olsen method, P2O5), 670.8 ppm exchangeable potassium (chloride of barium method), 1.22 % organic matter (Walchey-Black method), 17 % CaCO3 and 0.92 mmhos/cm electrical conductivity. This field belongs to semi-arid region. Experiments were conducted under rain fed conditions characterized by a sufficient and regular rainfall quantity. Weeds were manually controlled three times during each cropping season. Recommended fertilizer rate of N (Urea, 64 Kg/h) and P_2O_5 (DAP, 46 Kg/h) were applied to each plot in the shallow furrow depths and mixed with soil at the same time during sowing.

Each experimental trial was arranged on randomized complete block design comprising plots (six m rows, spaced 0.20 m apart), with three replicated checks for each variety. Sowing density was 350 plants per m^2. Weed sand diseases were controlled according to standard cultural practices. Plots were mechanically harvested at commercial maturity. After harvesting, the cleaned seeds were bulked and stored at 4°C until analysis. For the analysis of wheat varieties, the whole meal (particle size < 500 µm) was obtained by grounding wheat grains on a lab mill (RETSCH, Germany).

Meteorological data

The meteorological data of the sets are presented in (Table 1, Figure 1). Experiments were conducted at Kef (semi-arid region, Tunisia) during 3 seasons (2011-2012, 2013-2014 and 2014-2015). The annual maximum temperatures increased significantly, from season 2011-2012 to 2014-2015, whereas the minimum temperature variation was not significant (Table 1). The annual accumulated precipitation variation showed a significant decrease. Average climate data relative to grain-fill period showed significant variance. Season 2013-2014 maximum temperature was higher than in the two other seasons. In addition, season 2014-2015 average minimum temperature was the lowest. Accumulated precipitation during grain fill period was significantly lower in season 2014-2015 than in growing seasons 2011-2012 and 2013-2014.

Standardized static *in vitro* digestion method

The ground whole wheat samples were digested following the standard *in vitro* method [23]. Briefly, 1 g of sample was incubated 2 min with 1 ml simulated saliva containing amylase (75 U/ml of digesta); then, 2 mL of simulated gastric juice containing pepsin (2000 U/mL of digesta) were added and the sample was incubated for 2 h after adjusting the pH to 3. Subsequently, 4 mL of duodenal juice containing pancreatin (100 U trypsin activity/mL of digesta) and bile (10 mmol/L in the total digesta) were added and incubated for 2 h after adjusting the pH to 7. All the digestion steps were carried out at 37 °C under constant gentle mixing. Then, to inactivate the enzymes, the sample was boiled for 10 min at 95°C. After centrifugation (3220*g*, 4°C, 45 min), 295 µl of each sample supernatant was added to 5 µl of internal standard solution (LQLQPF(d_5)PQPQLPY, 0.41 mmol L^{-1}).

UPLC/ESI-MS analysis

UPLC/ESI-MS analysis was performed [24]. Briefly, the complex mixture obtained from enzymatic cleavage is separated by a RP column (ACQUITY UPLC BEH 300, C18, 1.7 mm, 2.1*150 mm; Waters corp., Milford, MA, USA) in a UPLC/ESI-MS system (Acquity Ultra-performance UPLC with a single quadrupole mass spectrometer; Waters SQD) using a gradient elution. Eluent A is a bi-distilled water solution with 0.1% formic acid (>99%) and acetonitrile (0.2%), and eluent B is an acetonitrile solution with 0.1% formic acid (>99%). Gradient elution was carried out as follows: 0-7 min 100% eluent A; 7-50 min from 100% to 50% eluent A; 50-52.6 min 50% eluent A; 52.6-53 min from 50% to 0% eluent A; 53-58.2 min 0% eluent A; 58.2-59 min from 0% to 100% eluent A; 59-72 min 100% eluent A. The samples are analyzed with UPLC/ ESI-MS in the Full Scan mode. Flow is 0.2 ml/min; analysis time 72 min; column temperature 35°C; sample temperature 18°C; injection volume 2 µL; acquisition time 7-58.2 min; ionization type is positive ions; scan range 100-2000 m/z; capillary voltage 3.2 kV; cone voltage 30 V; source temperature 150°C; desolvation temperature 300°C; cone gas flow 100 l/h; desolvation gas flow 650 l/h.

Data processing

The areas of the identified peptides and internal standard LQLQPF(d_5)PQPQLPY were integrated with the MassLynx software. The quantification value was obtained as the ratio peptide area/internal standard area multiplied by the moles of internal standard, assuming a response factor of 1. The result is reported on g of whole wheat flour considering the different dilution factors.

The identified gluten epitopes were subdivided into two groups: immunogenic peptides (sum of the amounts of the 8 identified immunogenic peptides obtained) and toxic peptides (sum of the amounts of the 8 identified toxic peptides obtained). The sum of immunogenic and toxic peptides is called the total immunogenic-toxic peptides.

Statistical analysis

For all the peptides identified, the analysis of variance (ANOVA) was performed. Significant differences among the mean values were calculated using Duncan's test. The fixed effect Multivariate Analysis of Variance (MANOVA) model was conducted twice. The first MANOVA included the main effects of genotypes, crop year and their interactions on 18 parameters (8 immunogenic, 8 toxic, total immunogenic, total toxic and total immune-toxic epitopes). The second MANOVA aimed to evaluate the effect of crop year, breeding history

and their interaction on 3 parameters (total immunogenic, total toxic and total immune-toxic epitopes). The percentage of total variation was computed to explain the variance of each epitope as a function of the main and interaction effects. All identified toxic and immunogenic peptides correlations were also calculated using Pearson's coefficient. Principal component analysis (PCA) was performed based on correlation matrix. The first two principal components were graphically represented in bi-plots. Clustering analysis was computed based on between group linkage method and squared Euclidian distance. All experimental data were statistically analyzed using the SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Identification of wheat peptides associated to CD using *in vitro* digestion

The Minekus and others method [23] of digestion has been established based on physiologically available and published *in vivo* enzyme and salt concentrations [25.] In this study, this static method was adopted to imitate durum wheat digestion. Peptides generated were analyzed by mass spectrometry (UPLC/ESI-MS) as shown in figure 2. The chromatogram might be subdivided into 2 phases; the first one (from 12 min to 30 min), where peptides ranging from Mr 200 to 3600 are eluted, whereas for the second phase (after 35 min), bile salts are eluted. It should be taking into consideration that the first 7 min of chromatographic run were excluded because they were rich in salt and sugars. These peptides are presented in Table 2. Peptides related to CD obtained derive mainly from gliadins (γ-gliadins, α-gliadins) and glutenin (LMW). Indeed, several peptides derived from various gluten proteins, including α- and γ-gliadins and recently glutenins have been reported to stimulate CD4+ T lymphocytes selectively isolated from small intestinal mucosa of CD patients [13, 16, 26, 27]. Our results showed that immunogenic peptides were exclusively γ-gliadins (8 immunogenic peptides identified, Table 2). We noted that γ-gliadins identified peptides contained a sequence motif QPQQPF, which has previously been identified by means of random phage cloning with sera from patients with CD [28], such as the two peptides IP7 and IP8, as previously found by Prandi and others [29], in the case of physiological digestion.

Peptides identified in the digestates as toxic sequences were mostly α-gliadin (8 toxic peptides identified, Table 2). RPQQPYPQPQPQ, from α-gliadin, was generated which is a toxic peptide in concordance with Cornell and others [30]. According to our data, LGQQQPFPPQQPYPQPQPFPS was identified as major toxic peptide. α-gliadin 31–49 (toxic

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core LGQQQPFPPQQPY) do not stimulate small intestinal T cells but cause *in vitro* and *in vivo* celiac toxicity [31]. Furthermore, in consistent with our findings, it reported also that the epitope QQQPP, repeated multiple times in the LMW glutenin sequence, has been shown to be the minimum IgE-binding sequence *in vitro* 19. Indeed, the key sequences of four amino acids associated with toxic fractions prepared from A-gliadin, these being QQQP and PSQQ [32].

Overall, UPLC-MS showed high sensibility in identifying and quantifying gluten epitopes. Indeed, several methods were previously adopted because they showed interesting potential in proteins identification but not in peptides. For instance, SDS-PAGE sensitivity was not sufficient to discriminate tiny difference in the gluten epitopes. Further, relaying on gluten or protein contents to estimate the allergenicity of wheat is not enough because gluten epitopes showed weak association with gliadin, total protein content, and no association with glutenins and gluten proteins [24]. Besides, unlike protein distribution, gluten epitopes showed important variability suggesting that two varieties might have similar protein or gluten content but not necessarily similar peptide content [24].

Genotype, crop year and their interactions influence on CD related epitopes

To recognize similarities in durum wheat allergens related to CD, a multivariate analysis was performed on 100 Tunisian durum wheat accessions grown during three crops seasons in the same location. The amounts of 8 immunogenic peptides, 8 toxic peptides, total immunogenic peptides, total toxic peptides and total immune-toxic peptides were subjected to MANOVA using Pillai's trace test. MANOVA results (Table 3) showed those allergen amounts were significantly affected by genotype (G), crop year (CY) and their interaction. The major determinant factor was genotype (Pillai's trace value=13.03, F = 13.03, P<0.001), in agreement with Prandi and others results [24,33]. G×CY interaction effect was found highly significant on CD-related peptide amounts (Pillai's trace value=7.02, F = 2.17, p<0.001). However, crop year factor (season 2014-2015, season 2013-2014 and season 2011-2012) was found the slightest effective parameter influencing wheat allergenicity (Pillai's trace value=1.39, F = 10.03, p<0.001). Even though genotype was the main parameter controlling immunogenic and toxic peptides (accounting for 62.87% and 64.71% of the total variance, respectively) (Table 3), we could not overlook the effects of crop year and G×CY interaction on durum wheat allergenicity.

Relative to genotype influence on CD-related peptides content, ANOVA results indicated that genotype had a highly significant effect. The total content of immunogenic peptides varied from a minimum of 372 ppm (Maali, modern accession, set 3) to 1442 ppm (EC Arbi, old accession, set 1) (mean value= 675 ppm ±86). The content of toxic peptides was ranging from 243 ppm (Maali, set 3) to 1050 ppm (EC Arbi, set 1) (mean value= 591 ppm ± 168). A high variability in the content of immunogenic and toxic peptides was found reflecting a large variation among the studied genotypes. Moreover, total immune-toxic peptides ranged from 615 to 1289 ppm (mean value=1278 ppm±371). This important range of variability could be attributed to high genetic diversity in durum germplasm released during the last century in Tunisia. Wheat samples belonged to landraces, old–intermediates and advanced cultivars, as explained on the plant material section.

Gluten epitopes that derived from γ-gliadin were more abundant than peptides derived from α-gliadin, in agreement with the results of Prandi et al. [29] in the case of physiological digestion. Additionally, *in vivo* trials confirmed that in several specific cohorts, a high frequency of CD patients was observed that mainly reacted to γ-gliadin peptides [27]. Concerning the immunogenic peptides, IP2, IP4 and IP6 had the highest values, while IP8 had the lowest one. QQPQQPFPQ was recurring epitope in the immunogenic peptides identified sequences. The repetitive presence of these residues makes the peptides a preferred substrate of tTG [13]. RPQQPYPQPQPQ (α-gliadin) was the major toxic peptide identified, similarly to previous findings [32].

As for crop year impact on wheat pathogenicity, analysis of variance revealed that allergens amount involved in CD varied in relation to the growing season exception for IP7. Environmental conditions, particularly fertilizer and temperature, were suggested to affect the amount, composition and/or polymerization of the gluten proteins [34]. Immunogenic peptides seasonal mean ranged from 519 ppm (season 2011-2012) to 735 ppm (season 2014-2015), while toxic peptides ranged from 400 ppm to 643 ppm. Total allergens were estimated to increase significantly from crop season 2011-2012 to crop season 2014-2015. The lowest value (920 ppm) was observed in season 2011-2012 with respect to both seasons (2013-2014) and (2014-2015) (1172 ppm and 1378 ppm, respectively). Our results showed an increase in immunogenic peptide amounts during the driest year (2014-2015). Similar trends were showed in toxic glutenin (IP7 and IP8). Duncan' test (Table 4) showed no significant difference between the two crop seasons (2014-2015 and 2013-2014) in IP1, IP2, IP3, TP1, TP5 and TP6. Similarities between results of both years could be attributed to comparable

annual water availability in both seasons, whereas dissimilarities in climatic data of grain fill period were responsible of the significant differences in total immunogenic-toxic peptides of these two seasons. These findings could suggest that some immunogenic and toxic peptides were affected by water availability during fill period. Thus, the highest response was observed in the crop year with the highest annual temperature and the lowest grain fill period accumulated precipitation. These findings could imply a high correlation between allergen amounts and climatic data. Although the trial fields were in the same semi-arid region under rain fed regime, high variability was shown confirming the relevant associations between water availability, temperature and gluten epitopes amounts.

G×CY interaction effect was highly significant for all the peptides except for IP3 and TP1 (Table 3). To further understand this interaction, a second MANOVA was performed. The influence of crop year (CY), breeding programs (BP) (abandoned and modern genotypes) and their interactions were studied on total immunogenic, total toxic and total immune-toxic peptides. Results revealed that crop year, breeding programs and their interactions effects were highly significant ($P < 0.001$). Pillai's trace test indicated a major impact of CY (Pillai's trace value=0.999, $F = 2630.60$, $p<0.001$), in comparison with BP (Pillai's trace value=0.993, $F=820.50$, $p<0.001$) and CY×BP interaction (Pillai's trace value=0.993, $F=415.98$, $p<0.001$).

Relative to the interaction CY×BP (Table 5), higher value in total immunogenic peptides was observed in abandoned lines grown in season 2014-2015 with respect to the ones grown in the two seasons 2013-2014 and 2011-2012. Similar trend was shown for toxic peptides. In 2014-2015 crop seasons, abandoned varieties' total immunogenic and immune-toxic peptides were slightly higher than modern genotypes (Table 5), in contrast with the other two seasons. In 2011-2012, important difference in total immune-toxic peptides was observed between abandoned lines and modern lines. Thus, a clear raise of allergenicity from abandoned to modern lines was showed under favorable rain fed conditions. Conversely, relative to season 2014-2015, Duncan' test revealed that there was no significant difference in epitopes amounts between abandoned and modern lines. This result demonstrated that severe rain fed conditions affected abandoned and modern genotypes pathogenicity in a similar way. We could attribute this result to resemblance in protein accumulation mechanism that probably occurred similarly under severe grain fill period underlining the deficit in water availability effect. Eventually, although there were significant differences between old and new cultivars, there was no constant pattern from year to year. Therefore, we could not conclude that there was an augmentation of gluten peptides amounts in durum wheat lines released during the 20th

century in Tunisia under rain fed conditions. As a matter of fact, no clear evidence of an increase in the gluten content of wheat was found in the United States during the 20th century, and if there has indeed been an increase in CD during the latter half of the century, wheat breeding for higher gluten content does not seem to be the basis [21]. Although the rising prevalence of adulthood onset of CD can hardly be explained, it could be due to an increasing number of subjects that lost the immunological tolerance to gluten in their adulthood [22]. Moreover, our results showed that regardless of the significant difference between both groups grown in three crop seasons, modern genotypes showed more stability from year to year climatic variation than landraces. These findings indicated also that newly released genotypes allergenicity was less sensitive to climatic variations, which is a useful indicator for wheat breeders.

Statistical Interpretation of the Obtained Data

Correlations existing among allergenic peptides, were computed using Pearson' test. Results revealed high significant correlations between the studied peptides. Immunogenic peptides were highly correlated (r(IP1, IP2)=0.739; r(IP1,IP4)=0.740; r(IP3,IP4)=0.807; r(IP3,IP5)=0.833; r(IP4,IP5)=0.909 and r(IP5,IP8)=0.81, $p<0.01$) due to the repeat motif of 17 amino acids (QQPQQPFPQQPQQPFPQ) [27.] However, a small number of significant correlations were observed between toxic peptides (r(TP2, TP5)=0,728 and r(TP3,TP4)=0,964, $p<0.01$). Furthermore, important correlation was shown between toxic and immunogenic peptides (r(TP2, IP4)=0,719; r(TP3,IP5)=0,790; r(TP3,IP6)=0,766; r(TP4,IP5)=0,819 and r(TP4,IP6)=0,796, $p<0.01$). IP, TP and TOT were also highly correlated to immunogenic peptides (IP3, IP4, IP5, IP6 and IP8) and toxic peptides (TP2, TP3, TP4 and TP 5).

Based on correlation results, a PCA was performed to enable an overview of immunogenic and toxic peptides associated with CD response to genotype and growing seasons. The first two components of the PCA (Figure 3a) explained 72.32% of the total variation (PC1, 50.95%; PC2, 21.37%). The first factor was related to immunogenic γ-gliadin (IP3, IP4, IP5, IP6 and IP8), toxic α-gliadin (TP3 and TP4), total immunogenic, total toxic and total immune-toxic amounts which were gathered on the right site of PC1. As for the second factor, immunogenic γ-gliadin (IP1 and IP2), toxic α-gliadin (TP1 and TP2) and toxic γ-gliadin (TP5) were located on the upper site, while toxic LMW (TP6, TP7 and TP8) were on the opposite site of PC2. PCA biplots enhanced a clear visualization of the classification of 100

accessions. The results of PCA allowed the clustering of 5 groups (Figure 3b) from the lowest immune-toxic group (Group 1) to the highest immune-toxic one (Group 5) as well as by clustering analysis:

Group 1 comprised 8 accessions which were 6 abandoned lines ((Mahmoudi (set3), Bidi (set3), Chili (set3), Richi (set 3), INRAT 69(set3), RVA Aoudy (set 1)) and 2 modern lines (Om Rabia (set3) and Salim (set3)).

Group 2 comprised 27 accessions: 19 abandoned lines (Biskri (set3), RVA Biskri Pub (set1), Bidi (set1and set2), RVA Azizi (set1), Kyperounda (set3), INRAT 69 (set2), Badri (set1), RVA Agili (set1), GT8 (set1), Jneh khottifa (set2 and set3), EC Jneh khottifa (set 1), RVA INRAT69 (set1), GT4 (set1), EC INRAT69 (set1), Mahmoudi (set2), Sbei (set1) and Maghrbi djerba (set1)) and 8 modern lines (Karim (set1 and set2), Khiar (set2 and set3), Nasr (set3), Maali (set 2 and set3) and EC Nasr (set1)).

Group 3 consisted of 47 accessions : 36 abandoned lines (EC Swabaa Eljia (set1), EC Biadha (set1), EC Mahmoudi (set1), EC Roumani (set1), GT6 (set1), RVA chetla (set1), EC Derbassi (set1), Khmiret zarzis (set1), EC Hmira (set1), EC Derbassi (set1), RVA Chili (set1), RVA Sbei (set1), EC Wared libled (set1), Hamira (set2 and set3), RVA Roumani (set1), Kyperounda (set1 and set2), Chili (set2), RVA Souaba Algiaa (set1), EC Oudi (set1), Biskri (set2), EC Chili (set1), Mahmoudi (set1), Jneh khottifa (set1) ,GT7 (set1), RVA Richi(set1), Bidi 17 (set1), Richi (set2), EC Biskri (set1), GT5 (set1), GT1 (set1), Aouij (set1), EC Azizi (set1), Biskri (set1), Azizi (set1) and 11 modern lines (EC Maali (set1), Waha1 (set1), Waha2 (set1), Nasr (set1 and set2), Om Rabia (set1and set2), Salim (set2), EC Khiar (set1), Maali (set 2) and Karim (set3))

Group 4 made of 16 accessions: 14 abandoned lines (RVA Ward el bled (set1), RVA Bidi Ap4 (set1), RVA Jneh khottifa (set1), EC Agili (set1), RVA Bayada (set 1), RVA Agile glabre (set1) ,GT2 (set1), Hmira (set3), Chili (set1), EC Richi (set1), RVA Arbi (set1), EC Bidi (set1), RVA Hmira (set1) and RVA Mahmoudi (set1) and 2 modern lines (Khiar (set1) and EC Om Rabia (set1))

Group 5 was formed by the old-line EC Arbi, with the most extreme location.

PCA screening revealed that the impact of the crop seasons was observed along with the first component (PC1), from crop year 2011-2012 with the lowest value to crop year 2014-2015 with the highest value. Moreover, the PCA biplots confirmed high genetic diversity in the

germplasm released during the last century in Tunisia, especially landraces compared to modern accessions. However, genotypes distribution in the factorial space could not confirm that breeding program enhanced the increase of celiac epitopes contrary to the controversial hypothesis suggesting ancient grains might show lower immunogenic properties [31]. In fact, in a recent CYMMIT wheat discussion paper, it was concluded that the species *T. monococcum* (cultivated einkorn), and other landraces, or "old modern wheat", of *T. aestivum*, *T. compactum* and *T. spelta* in which the wheat we eat today originated also contain gliadins (gluten proteins) like those in modern wheat that generate the gliadin peptides (epitopes) that provoke celiac disease [35]. Heterogeneous intestinal T-cell responses to ancient and modern wheat accessions were observed, additional evidence for the necessity of a strict lifelong gluten-free diet in CD patients [36.] Likewise, it was suggested that Graziella Ra and Kamut, two ancient durum wheats, are potentially as toxic as modern wheats regarding CD and strongly recommend that they should not be introduced in the diet of celiac patients [31]. Nevertheless, it was concluded that the pattern of *Triticum monococcum* gliadin proteins is sufficiently different from those of common hexaploid wheat to determine a lower toxicity in CD patients following in vitro simulation of human digestion [37].

This study was performed to access CD epitopes in Tunisian durum wheat historical series, to statistically study genotype and crop year influence and to compare CD epitopes of abandoned and modern cultivars. Using UPLC/ESI-MS technique, 16 immunogenic-toxic epitopes related to CD were identified, which were deriving from gliadins and glutenin (LMW): eight immunogenic peptides were exclusively γ-gliadins epitopes, while eight toxic peptides were four α-gliadin, one γ-gliadin and three LMW epitopes. The first MANOVA showed that genotype was the major factor controlling immunogenic and toxic peptides compared to crop year and their interaction. It has been demonstrated also a high variability in the content of immunogenic and toxic peptides reflecting an important genetic diversity in the studied germplasm. In addition, our finding showed important variability in CD epitopes due to year-on year climate variations associated mainly to accumulated precipitation during the grain fill period. The second MANOVA revealed that despite there were significant differences between abandoned and modern cultivars, there was no constant pattern from year to year in epitopes amounts. PCA and clustering analysis allowed the classification of 100 accessions in five groups from the lowest immune-toxic group to the highest immune-toxic one on the one hand; it confirmed the high genetic variance from old to new lines as function of growing year on the other hand. However, our findings were unable to confirm if new

breeding programs increased allergens amounts related to CD in durum wheat germplasm released during the 20th century in Tunisia.

Acknowledgments

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Figure caption

Figure 1: Accumulated precipitation; maximum and minimum temperature for the three growing seasons (2011–2012 (a); 2013–2014 (b) and 2014–2015 (c)); Acc-P: accumulated precipitation; Min-T: minimum temperature; Max-T: maximum temperature; ↓: grain filling period. This graph allowed a clear understanding on the difference between the three seasons in terms of temperature and precipitation. A highlight was attributed to filling time period due its important influence on the grain characteristics.

Figure 2: UPLC/ESI-MS chromatogram (total ion current) of a wheat sample. The chromatogram might be subdivided into phases: the first one (from 12 min to 30 min), where peptides ranging from Mr 200 to 3600 are eluted, whereas for the second phase (after 35 min), bile salts are eluted.

Figure 3: Principal components analysis of durum wheat epitopes involved in CD; (a): Biplot of principal component analysis, (b)**:** Rotated principal scores of the wheat accessions projected onto the first two principal components. The first two components of the PCA (Figure 3a) explained 72.32% of the total variation (PC1, 50.95%; PC2, 21.37%). The first factor was related to immunogenic γ-gliadin (IP3, IP4, IP5, IP6 and IP8), toxic α-gliadin (TP3 and TP4), total immunogenic, total toxic and total immune-toxic amounts which were gathered on the right site of PC1. As for the second factor, immunogenic γ-gliadin (IP1 and IP2), toxic α-gliadin (TP1 and TP2) and toxic γ-gliadin (TP5) were located on the upper site, while toxic LMW (TP6, TP7 and TP8) were on the opposite site of PC2. PCA allowed the clustering of 5 groups (Figure 3b) from the lowest immune-toxic group (Group 1) to the highest immune-toxic one (Group 5) as well as by clustering analysis.

Table 1: Meteorological data relative to annual and grain fill period of three growing seasons

	2014-2015		2013-2014		2011-2012	
	AAV	TGP	AAV	TGP	AAV	TVP
Min-T (°C)	9.2	5.9	9.3	6.9	9.2	8.3
Max-T (°C)	24	19.1	22.1	24.3	21.1	20.4
Acc-P (mm)	376.2	0.8	389.4	9.4	570	40.2

AAV: Annual average Value (October - July): TGP: till grain period; Acc-P: accumulated precipitation; Min-T: minimum temperature; Max-T: maximum temperature

554 **Table 2:** Immunogenic and toxic peptides identified in the digested samples

Code	Immunogenic peptides identified	Protein	Rt (min)
IP1	TQQPQQPFPQ	γ-gliadin	21.2
IP2	SQQPQQPFPQPQ	γ-gliadin	21.9
IP3	QAFPQQPQQPFPQ	γ-gliadin	25.0
IP4	TQQPQQPFPQQPQQPFPQ	γ-gliadin	25.5
IP5	PQTQQPQQPFPQFQQPQQPFPQPQQP	γ-gliadin	27.4
IP6	FPQQPQLPFPQQPQQPFPQPQQPQ	γ-gliadin	29.8
IP7	QPQLPFPQQPQQPFPQPQQPQQPSPQSQQPQQPFPQ	γ-gliadin	30.0
IP8	QQPQQPFPQPQQTFPQQPQLPFPQQPQQPFP	γ-gliadin	31.1
	Toxic peptides identified		
TP1	LQPQNPSQQQPQ	α-gliadin	17.2
TP2	RPQQPYPQPQPQ	α-gliadin	18.0
TP3	LQPQNPSQQQPQEQVPL	α-gliadin	24.6
TP4	LGQQQPFPPQQPYPQPQPFPS	α-gliadin	27.7
TP5	SQQQQPV	γ-gliadin	14.9
TP6	QQQPL	LMW-glutenin	17.2
TP7	QQQPPFS	LMW-glutenin	20.7
TP8	QQQPLPL	LMW-glutenin	26.1

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Table 3: F significance level and sum square percent of G, CY and G ×CY of Tunisian durum wheat identified allergens related to CD

		G		CY		G * CY	
	Immunogenic peptides identified	**Sig**	**%SS**	**sig**	**%SS**	**sig**	**%SS**
IP1	TQQPQQPFPQ	***	63.18	***	9.75	***	27.07
IP2	SQQPQQPFPQPQ	***	69.60	***	8.66	***	21.74
IP3	QAFPQQPQQPFPQ	***	73.99	***	12.78	Ns	13.22
IP4	TQQPQQPFPQQPQQPFPQ	***	61.69	***	17.23	***	21.08
IP5	PQTQQPQQPFPQFQQPQQPFPQPQQP	***	63.43	***	18.09	***	18.49
IP6	FPQQPQLPFPQQPQQPFPQPQQPQ	***	78.04	***	10.68	***	11.28
IP7	QPQLPFPQQPQQPFPQPQQPQQPSPQS QQPQQPFPQ	***	87.16	Ns	0.67	**	12.17
IP8	QQPQQPFPQPQQTFPQQPQLPFPQQPQ QPFP	***	75.32	***	6.74	***	17.94
IP	Total immunogenic peptides	***	62.87	***	19.60	***	17.52
	Toxic peptides identified						
TP1	LQPQNPSQQQPQ	***	71 84	***	7.81	Ns	20.35
TP2	RPQQPYPQPQPQ	***	72.71	***	18.68	*	8.61
TP3	LQPQNPSQQQPQEQVPL	***	74.24	***	14.53	**	11.22
TP4	LGQQQPFPPQQPYPQPQPFPS	***	71.83	***	13.95	***	14.22
TP5	SQQQQPV	***	55.80	***	29.15	**	15.05
TP6	QQQPL	***	84.65	***	2.12	***	13.23
TP7	QQQPPFS	***	80.46	*	1.53	***	18.00
TP8	QQQPLPL	***	79.77	**	2.58	***	17.65
TP	Total toxic peptides	***	64.71	***	20.22	**	15.08
Tot	Total peptides	***	62.28	***	22.05	**	15.68

G, genotype; CY = crop year; n.s. = not significant. * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$, SS: sum of squares

Table 4: Crop year effect on total immunogenic, total toxic and total immunogenic-toxic peptides related to CD

Code	Immunogenic peptides identified	2014-2015	2013-2014	2011-2012
IP1	TQQPQQPFPQ	77 b	78 b	68 a
IP2	SQQPQQPFPQPQ	102 b	98 b	84 a
IP3	QAFPQQPQQPFPQ	43 b	40 b	30 a
IP4	TQQPQQPFPQQPQQPFPQ	225 c	201 b	168 a
IP5	PQTQQPQQPFPQFQQPQQPFPQPQQP	118 c	95 b	78 a
IP6	FPQQPQLPFPQQPQQPFPQPQQPQ	79 c	52 b	36 a
IP7	QPQLPFPQQPQQPFPQPQQPQQPSPQSQQPQQPFPQ	22 a	20 a	17 a
IP8	QQPQQPFPQPQQTFPQQPQLPFPQQPQQPFP	69 b	47 a	38 a
IP	Total immunogenic peptides	735 c	632 b	519 a
	Toxic peptides identified			
TP1	LQPQNPSQQQPQ	29 b	32 b	23 a
TP2	RPQQPYPQPQPQ	194 b	179 b	119 a
TP3	LQPQNPSQQQPQEQVPL	81 c	39 b	21 a
TP4	LGQQQPFPPQQPYPQPQPFPS	177 c	115 b	67 a
TP5	SQQQQPV	34 b	33 b	22 a
TP6	QQQPL	69 a	71 a	86 b
TP7	QQQPPFS	41 a	49 b	54 c
TP8	QQQPLPL	19 b	18 ab	14 a
TP	Total toxic peptides	643 c	540 b	400 a
Tot	Total peptides	1378 c	1172 b	920 a

Means of same row followed by different letters differ significantly ($p < 0.001$).

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Table 5: CY ×BP interaction influence on total immune, total toxic and total immunogenic-toxic peptides related to CD

	2014-2015		2013-2014		2011-2012	
	AG	**MG**	**AG**	**MG**	**AG**	**MG**
IP	745 a	744 a	614 a	648 b	530 a	652 b
TP	650 a	649 a	54 b	534 a	391 a	550 b
TOT	1394 b	1393 b	1160 a	1182 b	92 a	1201 b

AG: abandoned genotypes; MG: modern genotypes

Means of same row followed by different letters differ significantly ($p < 0.001$).

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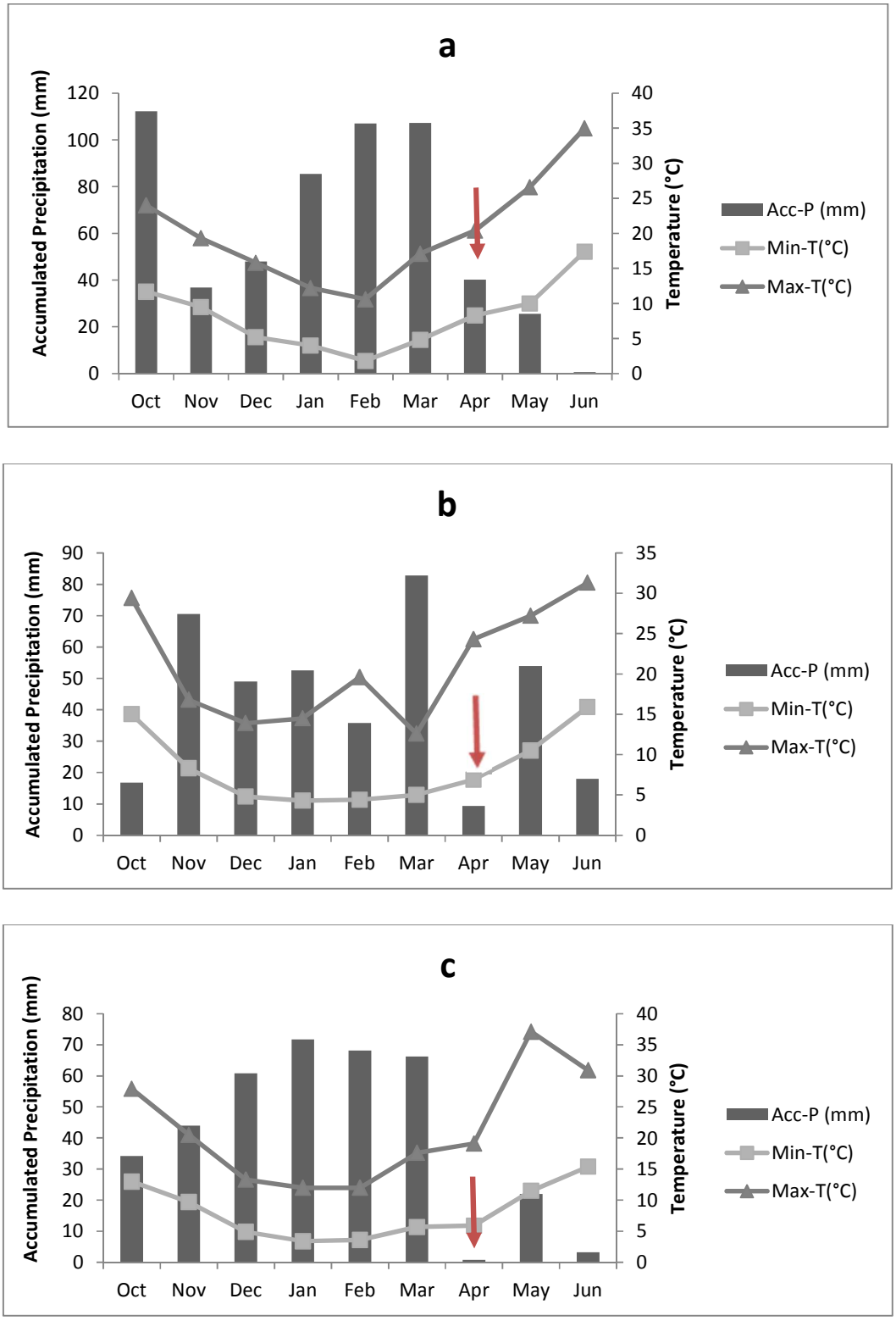
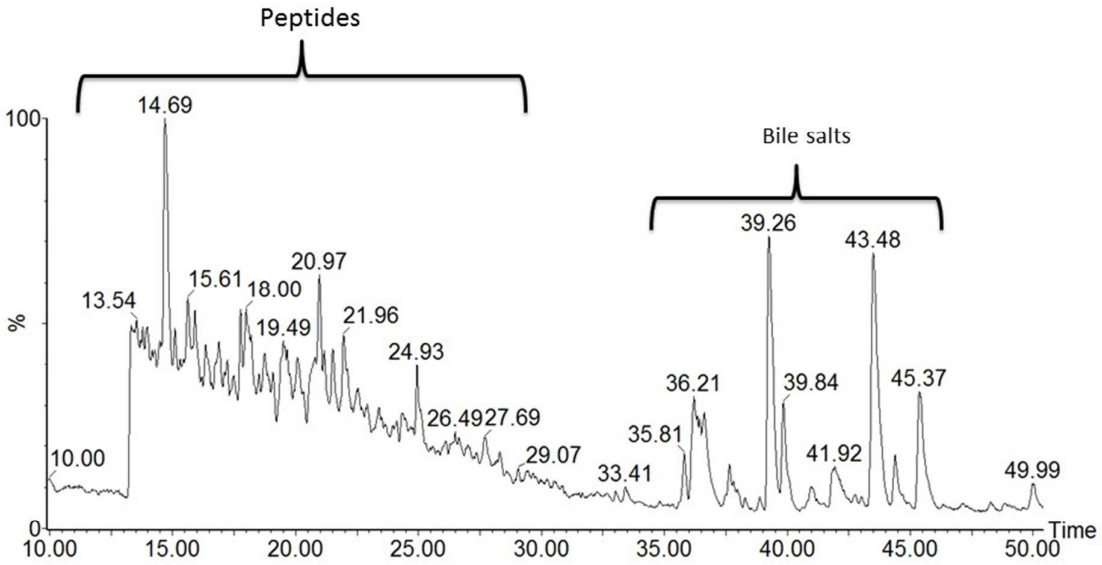


Figure 1: Accumulated precipitation; maximum and minimum temperature for the three growing seasons (2011–2012 (a); 2013–2014 (b) and 2014–2015 (c)); Acc-P: accumulated precipitation; Min-T: minimum temperature; Max-T: maximum temperature; ↓ : grain filling period.



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576 **Figure 2**: UPLC/ESI-MS chromatogram of a wheat sample.

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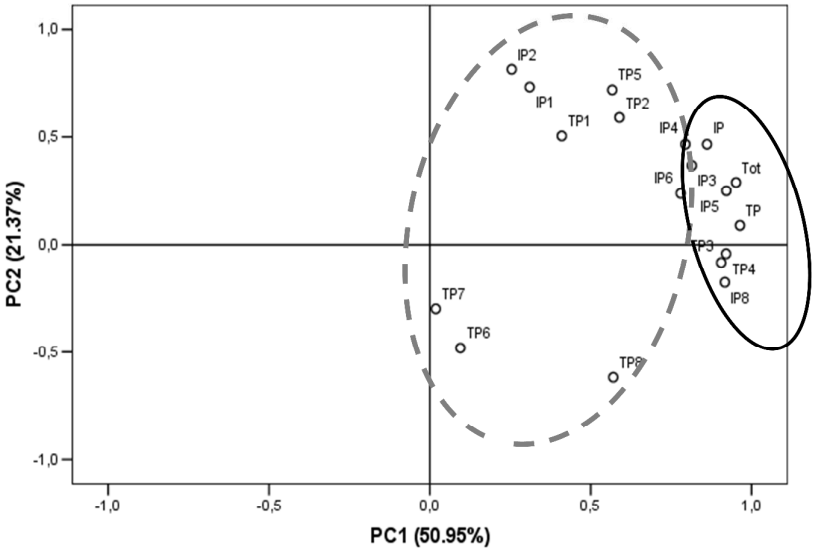
a



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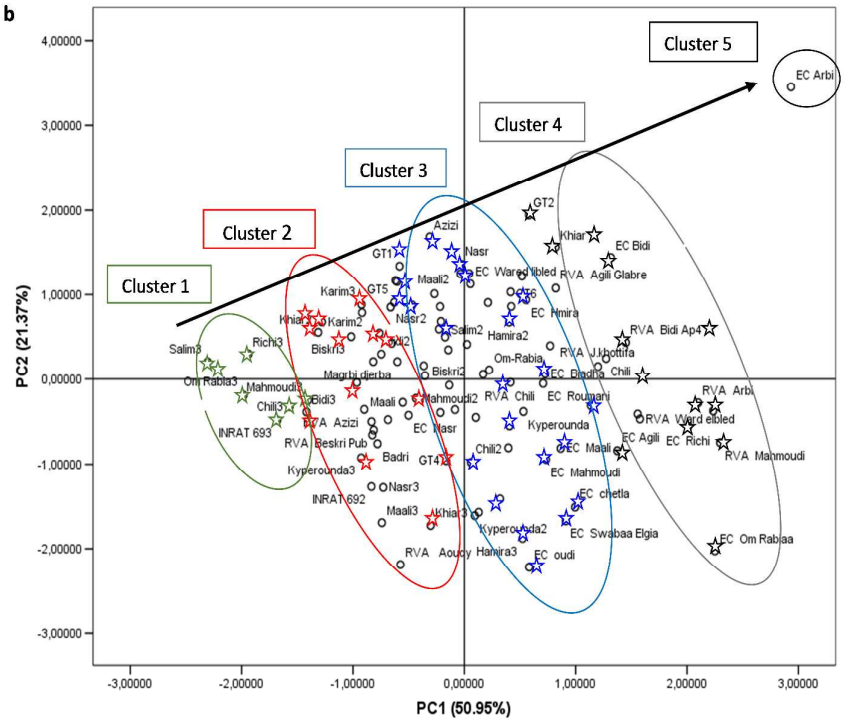
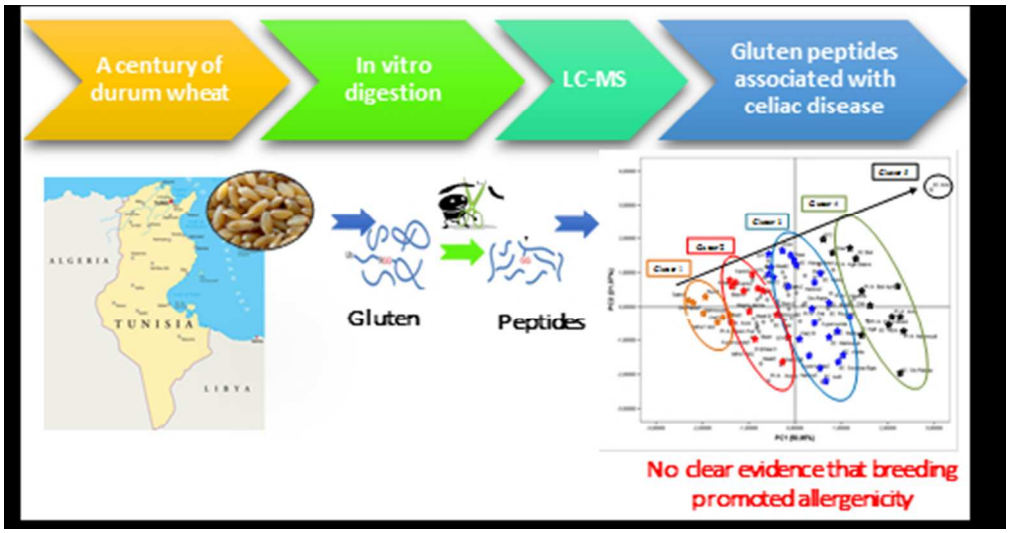


579

580 **Figure 3:** Principal components analysis of durum wheat epitopes involved in CD; (a): Biplot
581 of principal component analysis, (b)**:** Rotated principal scores of the wheat accessions
582 projected onto the first two principal components.



TOC Graphic

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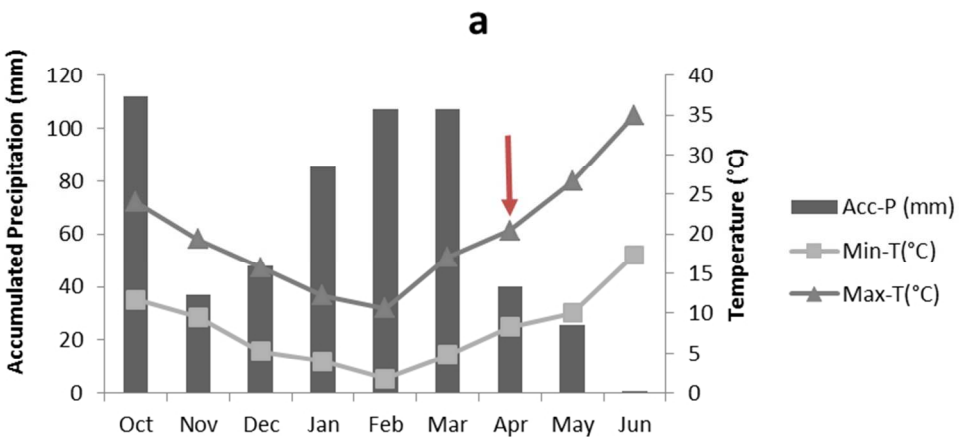

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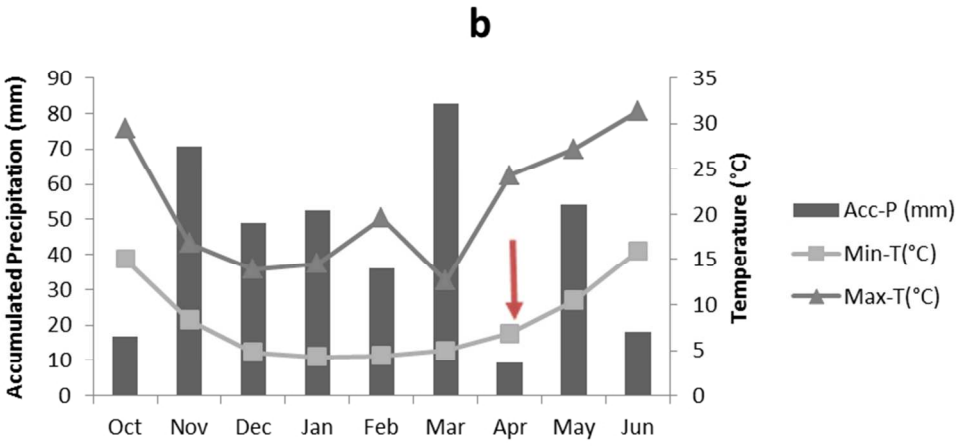

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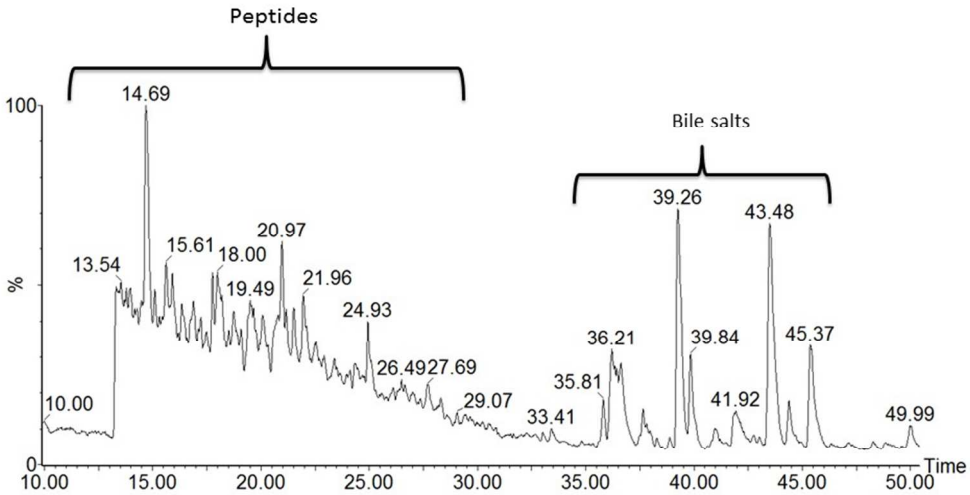

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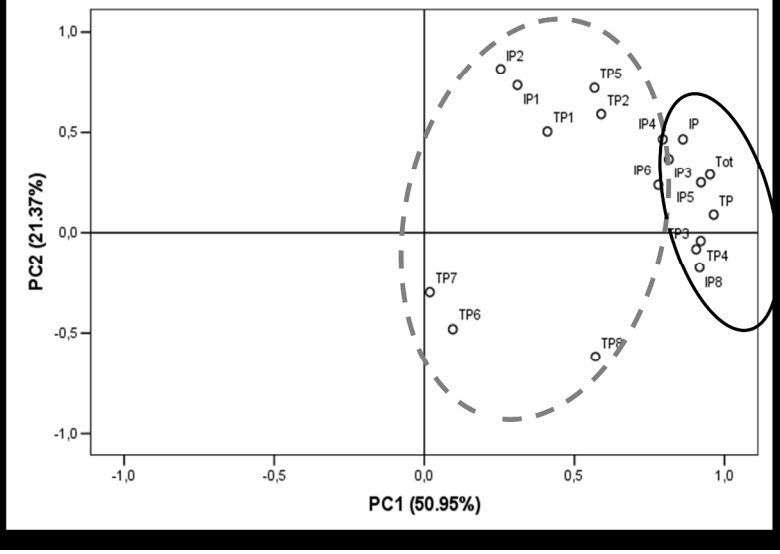

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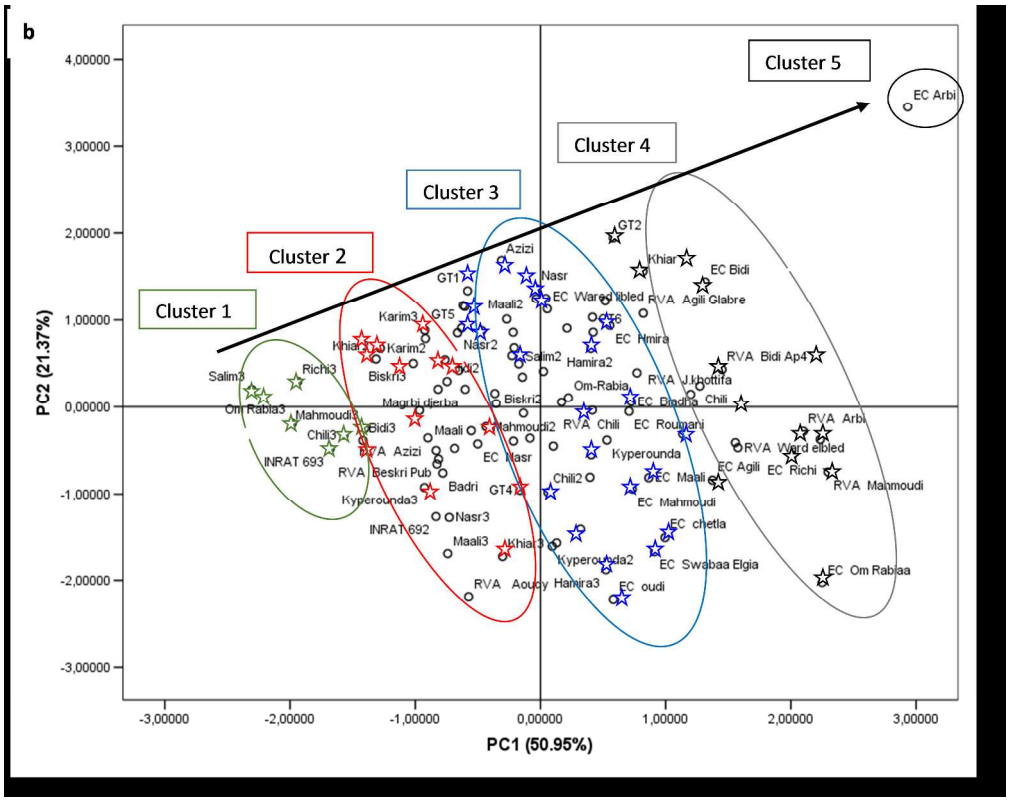

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