

Understanding the Effects of Genotype, Growing Year, and Breeding on Tunisian Durum Wheat Allergenicity. 1. The Baker's Asthma Case

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ABSTRACT: Baker's asthma is a serious airway disease triggered by wheat protein CM3 α -amylase/trypsin inhibitor. The purpose of the present study was to investigate the impact of genotype and crop year on allergen CM3 α -amylase/trypsin inhibitor associated with baker's asthma. A historical series of Tunisian durum wheat (100 accessions), derived from three crop years, was used to compare the amount of CM3 from landraces to advanced cultivars. CM3 protein quantification was assessed after an enzymatic cleavage of the soluble protein extracts on a UPLC/ESI-MS system, using a marker peptide for its quantification. Combined data analysis of variance revealed an important effect of genotype, crop year, and their interaction. The CM3 allergenic proteins were found to significantly vary among studied genotypes, as confirmed by genetic variability, coefficient of variance, heritability, and genetic advance.

KEYWORDS: CM3 α -amylase/trypsin inhibitor, baker's asthma, allergenicity, durum wheat

INTRODUCTION

Cereal researchers and breeders have focused on achieving food security, via improving yield rate, overlooking food safety. In the past, the safety of food did not emerge as a problem because the crop varieties had a long history of safe consumption.¹ Food allergies are one of the most serious food safety issues,² and in recent decades, there has been a mounting prevalence of allergies and certain intolerances, which are probably associated with the changes in consumers' lifestyle and food habits as well as the intervention of modern breeding.³

Allergic sensitization to wheat flour components is one of the most common triggers of occupational asthma.⁴ Baker's asthma is a serious work-related obstructive respiratory disease, which affects from 4% to 25% of bakery workers.⁵ However, the recent development and progress achieved in the field of allergy exposure and response might provide further clarifications to better understand baker's asthma.⁶ Indeed, it affects several professions dealing with wheat grain cultivation, transportation, production, and transformation. Although wheat is assumed to be the major trigger of baker's asthma, other grains, such as barley and rice, might be also involved.⁷

Several allergens, as well as some additives, have been involved in baker's asthma, but it remains that great focus was attributed to the water or salt soluble fraction of wheat proteins (albumins and globulins) as probably the major cause; indeed, the activity of specific IgE-binding accounts for about 70% to 80%.⁸ A wide spectrum of wheat grain proteins have been also identified to be involved with baker's asthma, such as serine proteinase inhibitors, thioredoxin, agglutinin, α - and β -amylases, peroxidase, acyl CoA oxidase, glyceraldehyde-3-

phosphate dehydrogenase, and triose phosphate isomerase.^{9–12}

More particularly, the α -amylase/trypsin inhibitor family is considered the major allergen of baker's asthma.^{13,14} Indeed, they are from 12 to 16 kDa polypeptides with 4 to 5 intrachain disulfide bonds responsible for their inhibitory activity.¹⁵ They are known as CM proteins because they are soluble in solution prepared by chloroform and methanol.¹⁶ Furthermore, to assess their activity, diagnostic tools are mainly skin prick tests, radioallergosorbent test, and immunoblotting assays.^{12,13,17}

Until now, few studies^{18,19} were performed on the immunochemical assessment of α -amylase/trypsin inhibitor CM3 allergenicity of wheat. In this research paper, we aimed to study the allergenic potential relative to the salt soluble albumins and globulins throughout the progress of breeding from landraces to high yielding genotypes of Tunisian durum wheat using many accessions grown in three growing seasons. For the furtherance of these aims, we sought first to identify asthma marker peptide for CM3 protein using the UPLC-MS technique, second to investigate allergenic variation during a century of breeding program in Tunisia, and ultimately to compare CM3 proteins in different wheat accessions grown in three different crop years.

MATERIALS AND METHODS

Description of Sampling. To study genotype and crop year influence on the content of the CM3 proteins associated with baker's

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asthma, representative durum wheat sets, released during the 20th century in Tunisia, were used. Three sets were grown during three seasons (2011–2012, 2013–2014, and 2014–2015) as mentioned below in detail.

Set 1 (season 2014–2015) comprises 70 accessions of durum wheat consisting of 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 five groups according to the year in which each cultivar was released:

- Group 1 (from 1900 to 1940) comprises 51 indigenous and exotic landraces: Hmira (4 accessions), Jenah Khottifa (3 accessions), Azizi (3 accessions), Aouij (1 accession), Sbei (2 accessions), Derbassi (2 accessions), Wared Lebled (2 accessions), Biadha (2 accessions), Swabaa Elgia (2 accessions), Echlatla (2 accessions), Roumani (2 accessions), Aoudy (2 accessions), Richi (2 accessions), Biskri (3 accessions), Agili (3 accessions), Arbi (2 accessions), Mahmoudi (3 accessions), Bidi (3 accessions), Bidi 17 (1 accession), and LS (7 accessions).
- Group 2 (from 1940 to 1970) comprises 7 old cultivars that were adopted from international programs (Chili (3 accessions) and Kyperounda (1 accession)) and varieties selected from crosses involving landraces (INRAT 69 (2 accessions) and Badre (1 accession)).
- Group 3 (from 1970 to 1980) comprises 1 CIMMYT genotype Maghrebi (1 accession).
- Group 4 (from 1980 to 1990) comprises 3 CIMMYT genotypes (Karim (1 accession) and Khiair (2 accessions)) and 6 ICARDA genotypes (Waha (2 accessions), Om Rabia (2 accessions), and Nasr (2 accessions)).
- Group 5 comprises 2 modern lines selected after the 1990s (Maali (2 accessions)).

Set 2 (season 2013–2014) comprises 15 accessions: 6 landraces (Hamira, Jenah Khottifa, Richi, Biskri, Mahmoudi, and Bidi), 3 old cultivars (Chili, Kyperounda, and INRAT 69), and 6 high yielding varieties that are results of crosses made in CIMMYT (Karim and Khiair), in ICARDA (Om Rabia and Nasr), and in INRAT (Maali and Salim).

Set 3 (season 2011–2012) comprises 15 accessions: 6 landraces (Hamira, Jenah Khottifa, Richi, Biskri, Mahmoudi, and Bidi), 3 old cultivars (Chili, Kyperounda, and INRAT 69), and 6 high yielding varieties that are results of crosses made in CIMMYT (Karim and Khiair), in ICARDA (Om Rabia and Nasr), and in INRAT (Maali and Salim).

Field Setup. Field experiments were carried out in the trial field of the Graduate School of Agriculture of Kef during three growing seasons (2011–2012, 2013–2014, and 2014–2015) on a clay-loam soil. Each experimental trial was arranged on randomized complete block design comprising plots (6 m rows, spaced 0.20 m apart), with three replicated checks for each variety.

Climatic Data. Rainfall distribution and temperature trends in the three years are presented in Figure 1. High significance differences in the trends of accumulated precipitation were shown during the grain filling period (April). Accumulated precipitation during April was high in season 2011–2012 (40.2 mm), moderate in season 2013–2014 (9.4 mm), and almost completely absent in season 2014–2015 (0.8 mm).

In the first year (2011–2012; Figure 1a) accumulated precipitation average value was the highest (570 mm). Important precipitation was marked mainly during autumn and winter. The second year (2013–2014; Figure 1b) was characterized by 389 mm (average accumulated precipitation), 9.3 °C (average minimum temperature), and 22.1 °C (average maximum temperature). Moderate rainfall was recorded during winter followed by a raise during March, while the last year (2014–2015; Figure 1c) was the driest. Also, considerable difference in the trends of temperature was shown in the three crop seasons, more specifically during the spring.

Salt Protein Extraction. Soluble proteins were extracted from wheat kernels (0.5 g) using 0.5 M NaCl aqueous solution (10 mL) for

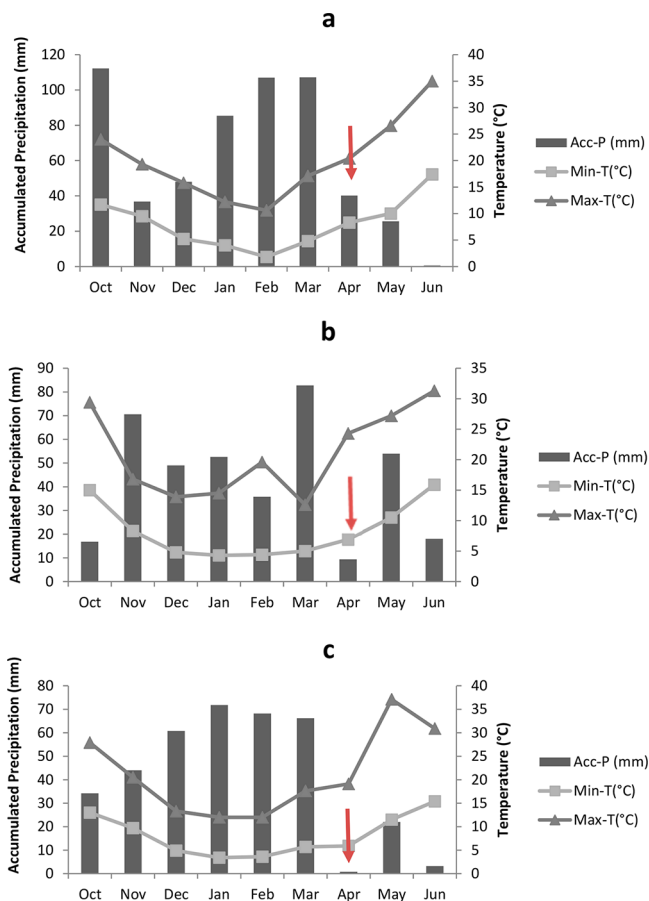


Figure 1. Accumulated precipitation; maximum and minimum temperature for the three growing seasons (2011–2012 (a); 2013–2014 (b); and 2014–2015 (c)); Acc-P, accumulated precipitation; Min-T, minimum temperature; Max-T, maximum temperature; ↓, grain filling period.

2 h and 30 min at room temperature. The supernatant was recuperated by centrifugation (3220g, 15 min, 4 °C), and then stocked at 20 °C.¹⁸

In Vitro Enzymatic Digestion. The enzymatic in vitro digestion was described by ref 18. In brief, the extract (1 mL) was dried under nitrogen flux, and then redissolved with 10 mM HCl aqueous solution (500 mL, pH 2). For the in vitro digestion, 1 mg/mL pepsin solution (20 μ L) was added and the mixture was incubated for 3 h at 37 °C. Then, 100 mM NaH₂PO₄ solution (300 μ L, pH = 7.2), 1 mg/mL chymotrypsin solution (20 μ L), and 1 mg/mL trypsin solution (20 μ L) were added and the mixture was incubated for 4 h at 37 °C. The samples were boiled in a water bath (5 min, 95 °C), dried under nitrogen flux, reconstituted with 0.1% formic acid solution (300 μ L), and centrifuged (15093g, 10 min, 4 °C). For the UPLC/ESI-MS, 250 μ L of supernatant were injected with 5 μ L of internal standard (FIA (d3) LPVSPQPDPR solution (0.214 mM)).¹⁸

Ultraperformance Liquid Chromatography (UPLC/ESI-MS) Analysis. The obtained mixture was separated by a RP column (Aeris peptide C18, 1.7 μ m, 150 $\times$ 2.10 mm) in a UHPLC/ESI-MS system (Dionex ultimate 3000 with a triple quadrupole Thermo TSQ vantage) using a gradient elution. Eluent A was bidistilled water with 0.1% formic acid and 0.2% acetonitrile, and eluent B was acetonitrile with 0.1% formic acid; gradient: 0–7 min 100% A, 7–50 min from 100% A to 50% A, 50–52.6 min 50% A, 52.6–53 min from 50% A to 0% A, 53–58.2 min 0% A, 58.2–59 min from 0% A to 100% A, 59–72 min 100% A. The digested sample extracts were analyzed by the mass analyzer running 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 μ L; acquisition time 7–58.2 min; ionization type positive ions; scan range 100–2000 *m/z*; spray voltage 3500 V,

Table 1. Sum of Squares from the Combined Analysis of Variance of 100 Durum Wheat Genotypes Grown in Tunisia during Three Growing Seasons^a

source	type III sum of squares	Df	mean square	F value	probability
corrected model	35017503.198	99	353712.15	234.12	0
intercept	40924950.13	1	40924950.13	27087.98	0
G	28676147.33	70	409659.25	271.15	0
CY	2571689.286	2	1285844.64	851.09	0
G × CY	4070191.667	27	150747.84	99.78	0
error	151081.608	100	1510.82		
total	100347838.3	200			
corrected total	35168584.81	199			

$R^2 = 0.996$ (adjusted $R^2 = 0.991$)

^aG: genotype. CY: crop year.

Table 2. Estimates of Components of Variance and Genetic Parameters for α -Amylase/Trypsin Inhibitors in Durum Wheat Accessions^a

	Vg	Ve	Vp	$\bar{x}$	GCV %	PCV %	ECV %	H ²	GA %
CM3 protein	43151.90	25124.64	68276.54	570.87	36.39	45.77	27.77	63.20	74.96

^aVp, Vg, and Ve: phenotypic variance, genotypic variance, and environmental variance. $\bar{x}$: grand mean of the population. PCV: phenotypic coefficient of variation. GCV: genotypic coefficient of variation. ECV: environmental coefficient of variation. H²: broad sense heritability. GA: genetic advance.

vaporizer temperature 250 °C; sheath gas pressure 22; auxiliary gas pressure 10; capillary temperature 250 °C.

Data Processing. The characteristic ions (695.6 *m/z*, 768.9 *m/z*, and 770.5 *m/z*) were extracted, obtaining extract ion chromatograms (XICs), in which the areas of the identified peptides and internal standard FIA(d3)LPVSPQPDPR were integrated with the Thermo Scientific Xcalibur software. The result was expressed as the ratio peptide area/internal standard area multiplied by the moles of internal standard.

Data Analysis. Statistical Analysis. The Statistical Package for the Social Sciences (SPSS for windows version 11.0; SPSS Inc., Chicago) was used for the statistical analyses. One-way analysis of variance (ANOVA) and combined analysis of variance were performed using SPSS software (SPSS, Inc.). Genotype was regarded as a fixed effect, while environment was regarded as a random effect. The level of significance was expressed as significant at $p < 0.05$ and highly significant at $p < 0.001$.

Estimation of Genetic Variability, Coefficient of Variance, Heritability, and Genetic Advance. Genotypic, phenotypic, and environmental coefficients of variation (GCV, PCV, and ECV) were computed.²⁰ Broad sense heritability was estimated as the ratio of genotypic variance (GV) to the phenotypic variance (GP) and expressed in percentage.²¹ Genetic advance (GA) was estimated assuming a selection differential at 5% level intensity (2.06).²² Genetic advance (GA %) was calculated as percentage of mean.²⁰

RESULTS AND DISCUSSION

Combined Analysis of Variance for CM3 Proteins of 100 Tunisian Durum Wheat Accessions. Combined analysis of variance was conducted to investigate genotype and crop year effects on CM3 amount (Table 1). Results revealed that α -amylase/trypsin inhibitors were significantly affected by genotype (G), crop year (CY), and G × CY interaction. However, in most cases, only single G or CY factors have been evaluated. Results suggested a significant G × CY interaction impact on the expression of allergen content in wheat genotypes. Likewise, these results revealed that durum wheat accessions react differently with the climate scenario of each growing season. Partitioning of corrected total sum of square (CTSS) indicated that the genotype effect accounted for 81.54% of the CTSS, G × CY interaction effect was 11.57%, and crop year effect presented 7.1% of CTSS. The greatest part of the CTSS was explained by the genotype effect, which might

be probably related to a genetic variability in the expression of the α -amylase/trypsin inhibitor CM3.¹⁸ Despite that all the field trials were conducted under rain fed conditions and in the same cultivation area, a significant impact of crop year was shown that could be explained by the different rainfall distribution patterns. Moreover, these findings suggested the hypothesis that allergenicity in durum wheat was highly correlated to G × CY interaction. G × CY interaction could be a valuable tool for breeding programs because it joins both genotype and crop year factors.

Genotype Effect on the Amount of Allergenic Polypeptides. *Variances, Coefficients of Variation, Heritability, and Genetic Advance of the Amount of Allergenic Polypeptides.* Results of ANOVA indicated a high significant variance ($p < 0.001$) in the content of pathogenic peptides generated, suggesting the existence of high genetic diversity among the genotypes. The content of peptides ranged from 92 ppm (Arbi, accession 2, old cultivar) to 2657 ppm (Agili, accession 1, landrace). Significant difference was shown among the different accessions such as the landrace Hmira (season 2014–2015); allergen content values in the three accessions (1, 2, and 3) were 158, 702, and 296 ppm, respectively. Problem of gene expression regulation could be the main factor for the ensuing difference.

Phenotypic, genotypic, and environmental variances and coefficients as well heritability and genetic advance are presented in Table 2. Results showed that environmental coefficient of variation (ECV) was lower than genotypic coefficient of variation (GCV). Moreover, phenotypic variance (PCV) was important for CM3 proteins. Genetic parameters were heritability, genetic gain, and genetic advance. By definition, heritability is the proportion of genetic variability passing from one generation to the next.²³ Estimates of heritability and genetic gain showed high heritability (63.20%), emphasizing that the CM3 content was basically under the leading influence of the genotype (45.77%) rather than the environment (27.77%). These findings could be valuable for future breeding programs. No doubt, G × CY interaction should be taken into consideration. It was established that heritability values together with genetic gain were more

valuable than heritability alone in predicting the effect of selection.²³ A high genetic gain value (74.96%) was recorded confirming as well the importance of genetic impact on CM3 allergen content.

Breeding Program Progress Impact on the Amount of Allergenic Polypeptides. Although breeding programs' influence on respiratory allergy is an important safety issue, Kucek et al.²⁴ inferred that there are no available comparative studies between heritage and modern wheat genotypes in terms of inhibitory activity against human enzymes. So, the scope of this section was to enable a clear understanding of the degree of breeding programs' impact on allergen content. Significant variation in CM3 proteins among the studied groups was revealed, as confirmed by ANOVA ($p < 0.001$). The use of box-plots (Figure 2) was an approach to illustrate allergen variance

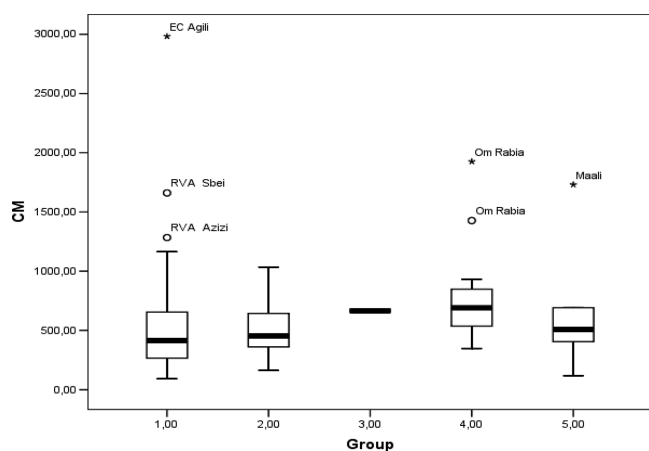


Figure 2. Boxplot of CM3 protein deviation for 100 durum wheat accessions during the five phases of breeding programs phase in Tunisia. Quartiles and median are used to construct the box. An individual box plot shows the requirement median (heavy vertical line); the 25th to 75th percentiles (the box itself); the range of the data (the "whiskers"); and potential outliers (*). 1: group 1. 2: group 2. 3: group 3. 4: group 4. 5: group 5. CM: CM3 protein content.

between the five different phases of wheat history in Tunisia. Landraces (group 1) and old cultivars (group 2) showed high range of variability, confirming the important genetic diversity of abandoned accessions compared to advanced ones. Landraces ranked fourth in CM3 protein content (mean value = 520 ppm). The lowest pathogenic old variety, Arbi (accession 2) (92 ppm), might potentially be developed for prevention for people who are genetically predisposed but still healthy. Old varieties selected from 1940 to 1970 (group 2) had slightly lower values (mean value = 517 ppm) than landraces. Then, a clear increase was observed for intermediate accession Maghrebi (666 ppm) selected in 1972 by CYMMIT (group 3). The most cultivated cultivars nowadays in Tunisia (group 4) had the highest allergen content (mean value = 825 ppm), reflecting the negative effect of breeding programs on wheat allergenicity, which raises the question of how the allergenicity is not yet controlled in the newly selected genotypes. Conversely, a decrease of allergenicity was recorded in varieties selected after 1990 in INRAT (group 5) (mean value = 661 ppm). Both genotypes Maali and Salim (group 5) were the results of crosses made in Tunisia between old lines and advanced lines. The pedigrees of Maali and Salim were (CMH80A.1060/4/T.TURA/CMH74A.370//CMH77.774/3/YAV79/5/RAZZAK/6DACK/YEL//KHIR) and (ALTAR84/

FD8419-126-1-2/Razzak/3/Krf/Baladia Hamra), respectively.²⁵

In the context of plant defense, many of the albumin and globulin proteins have important contributions in abiotic stress or biotic defense, such as amylases and amylase/trypsin inhibitors.²⁶ EC Agili (landrace) could be a good candidate for plant selection. Thus, CM3 protein studies are crucial to allow a better understanding of their role in plant defense mechanisms as well as human allergies, and consequently to orientate breeding strategies and biotechnological approaches for these specific focuses.²⁷

Crop Year Effect on the Amount of Allergenic Polypeptides. The year-on-year variations in climate showed significant impact on allergen content in each soluble protein fraction in this study. Results were reported in a box and whiskers plot (Figure 3). These graphs enabled the visualization of the

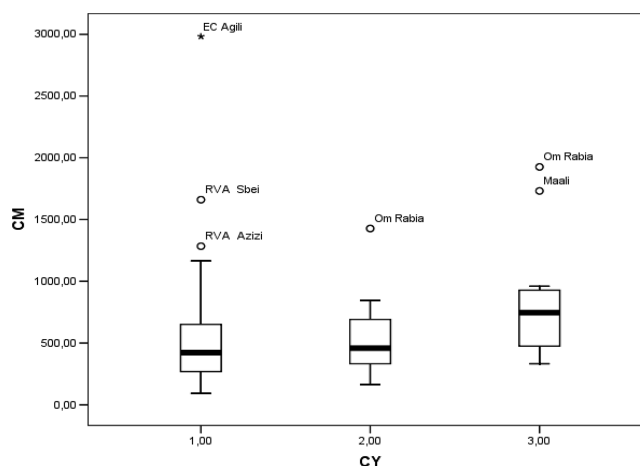


Figure 3. Boxplot of CM3 protein content for 100 wheat cultivars grown over three growing seasons (2011–2012/2013–2014/2014–2015). Quartiles and median are used to construct the box. An individual box plot shows the requirement median (heavy vertical line); the 25th to 75th percentiles (the box itself); the range of the data (the "whiskers"), and potential outliers (*). 1: season 2014–2015. 2: season 2013–2014. 3: 2011–2012. CY: crop year. CM: CM3 protein content.

distribution of the values of CM3 protein content of the 100 studied accessions. An overall box-and-whisker plot analysis indicated a high variability between the three crop years of sampling. Indeed, set 1 (season 2014–2015) showed the highest range of variability. CM3 allergen content ranged from 92 ppm (Arbi, accession 2, old cultivar) to 2657 ppm (Agili, accession 3, landrace). This variability could mainly be attributed to the important number of samples (70 accessions) and the wide range of breeding diversity. Set 2 allergen amounts varied from 163 ppm (INRAT69, old cultivar) to 1427 ppm (Om Rabia, modern genotype), whereas set 3 allergen range was from 332 ppm (Biskri, landrace) to 1926 ppm (Om Rabi, modern genotype). Additionally, set 3 (2011–2012 climate scenario) allergen content mean value was 818 ppm, while set 2 (2013–2014 climate scenario) and set 1 (2014–2015 climate scenario) mean values were 548 and 523 ppm, respectively. These results showed that the amounts of allergens were positively correlated to precipitation during grain fill period. This pattern could be the result of water availability rise during 2011–2012 crop filling phase of the grain. Indeed, high rainfall was registered during the season 2011–2012 (40.2

mm) compared to both seasons 2013–2014 (9.4 mm) and 2014–2015 (0.8 mm), as detailed in [Materials and Methods](#). Thus, accumulated precipitation during April could be directly implicated in the content of CM3 content. In fact, the albumin–globulin fraction accumulates from anthesis to ~20 days after anthesis, and after that they keep almost a constant level,²⁴ while the genetic and environmental mechanisms influencing the accumulation are still unknown.²⁸

A wide set of Tunisian durum wheat was the subject of an immunochemical study to assess the amylase/trypsin inhibitor CM3 amounts related to baker's asthma. Statistical results confirmed that genotype, crop year factors, and their interactions had a key role in determining wheat allergenicity. Furthermore, high variance and coefficient of variability at genotypic and phenotypic levels associated with a high heritability and genetic gain clearly indicated that the expression of these characters is under genetic control. Nonetheless high significant differences were observed between studied accessions, and rainfall patterns during grain fill period were found to have also an important influence on allergen content. Indeed, the decrease of the amounts of pathogenic peptides under severe rainfed condition confirmed the significant implication of growing seasons.

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Notes

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