

Changes in proteins and starch granule size distribution during grain filling of triticale

Pérez, G.T.; P.D. Ribotta, A.V. Aguirre, O.J. Rubiolo and A. E. León

SUMMARY

Changes in protein fractions and starch granule size distribution were evaluated throughout grain filling, from 14 to 47 days after anthesis (DAA) for two lines (T and B) of triticale (*X Triticosecale* Wittmack). Synthesis of the albumin-globulin fraction decreased during grain filling while prolamin and glutelin synthesis increased. Significant differences were found in protein content at different stages of synthesis between the two lines, T and B, the B line showing a faster rate of synthesis. As developmental grain approached physiological maturity, there was an increasing proportion of the large polymers separated under unreducing multi-stacking gel electrophoresis. The small polymer and protein subunit separated in 12% resolving gel decreased in T but did not change in B line from 14 DAA until physiological maturity. The size distribution of starch granules of both triticale lines was characterized during grain filling, by using a light-scattering particle size analyzer. A bimodal size distribution was found. The percentage volume of the large granule decreased and the small granule increased during ripening. In B line, starch granules were smaller and showed a higher percentage volume than those of the T line. The result observed in protein synthesis and granule size suggests that the differences between the two lines could be related with the high shriveling level of B line grains.

Keywords: grain-filling, triticale, starch granules, protein synthesis.

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RESUMEN

En el presente trabajo se evaluaron los cambios producidos en las fracciones

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proteicas y en la distribución de tamaño de los gránulos de almidón durante el llenado del grano de triticale. El seguimiento se realizó sobre dos líneas de triticale (*X Triticosecale* Wittmack) (T y B) desde el día 14 al día 47 después de anthesis (DAA). La síntesis de albúminas y globulinas disminuye a medida que el grano se va llenando, mientras que la de las fracciones de prolaminas y glutelinas aumenta. Se encontraron diferencias significativas en el contenido de proteínas entre las dos líneas en los diferentes estados, la línea B mostró una mayor velocidad de síntesis. A medida que los granos alcanzaron la madurez fisiológica, se observó un incremento en la proporción de grandes polímeros proteicos separados por electroforesis en geles de poliacrilamida en condiciones no reductoras. Las proteínas más pequeñas y las retenidas en el gel con una concentración del 12% de acrilamida, disminuyeron en la línea T, pero se mantuvieron sin cambios en B desde los 14 DAA hasta madurez fisiológica. La distribución de tamaño de los gránulos de almidón se caracterizó con un analizador de tamaño de partículas. En ambas líneas se observó una distribución de tamaño bimodal. Durante la maduración el volumen porcentual de los gránulos grandes disminuyó y el de los menores aumentó. En la línea B, los gránulos fueron más pequeños y mostraron un mayor volumen porcentual que en la línea T. Los resultados obtenidos en la síntesis de proteínas y en el tamaño de los gránulos de almidón, sugieren que las diferencias observadas entre las dos líneas pueden estar relacionadas con la mayor presencia de grano arrugado en la línea B.

Palabras clave: llenado de grano, triticale, gránulos de almidón, síntesis de proteínas.

G.T. Pérez; P.D. Ribotta; A.V. Aguirre; O.J. Rubiolo y A. E. León. Facultad de Ciencias Agropecuarias, U.N.C., CC 509, 5000 Córdoba, Argentina. (aeleon@agro.uncor.edu)

INTRODUCTION

Triticale (*X Triticosecale* Wittmack) is a hybrid resulting from the crossing between wheat (*Triticum* sp.) and rye (*Secale* sp.). In areas of the world where diseases or untoward soil conditions restrict wheat production, triticale has proved to be an alternative crop for human consumption.

Spring triticals are cultivated in Argentina. These cultivars usually have the rusticity and tolerance to weather conditions that are harmful to rye. However, good flour yield and plump kernel have not been obtained as yet. Several factors combine to determine flour yield: grain size and shape, grain hardness and grain shriveling (Baker and Golumbic, 1970; Marshall *et al.*, 1986). Grain shrivelling is an undesirable characteristic that results in low grain test weights and low flour yields (Peña and Bate, 1982; Peña and Amaya, 1992). This disadvantage is originated, after cell division, when proteins and starch are deposited in the endosperm which forms a starch protein matrix (Jönsson, 1987).

Up to now, in Argentina triticale has been used as a forage crop, but some cultivars have shown suitable characteristics for the production of flour for

cookies (León *et al.*, 1996; Rubiolo *et al.*, 1998; Aguirre *et al.*, 2002).

The triticale flours which have the best cookie quality exhibit low protein content, high prolamine percentage and high proportion of 34 kDa prolamine (León *et al.*, 1996).

Cereal storage proteins are typical secretory proteins. They are synthesized in ribosomes attached to the endoplasmic reticulum and pass into the lumen with the cleavage of an N-terminal signal peptide. Folding and disulfide bond formation, including the assembly of high M_r disulfide-bonded glutenin polymers are thought to occur within the endoplasmic reticulum lumen. The storage proteins are deposited in the developing endosperm cells in discrete protein bodies, which appear to coalesce during the later stages of development forming a continuous proteinaceous matrix surrounding the starch granule. This matrix is the origin of gluten. The assembly of gluten subunits into high M_r polymers appears to be highly sensitive to environmental factors. (Shewry, 1999)

In cereal endosperm, starch synthesis is initiated in the proplastids with the formation of one small

starch granule, which at first occupies only a small volume of this organelle. There is, then, a gradual increase in size of the granule until the mature amyloplast is completely filled. Wheat and other cereals contain both large and small amyloplasts in the mature endosperm. It appears that the large ones (A type) are initiated about 4-5 days after anthesis, the final number of A-type amyloplasts being achieved about 7 days later when cell division ceases (Bewley and Black, 1994).

The A type granules increase in size throughout kernel growth to final diameters which range from 10 μm to maximum diameters, variously reported as 36, 40 45 and 50 μm , there being evidence that the final granule size depends on the cultivar and season.

At maturity, A-type starch granules comprise 3% of the total number of granules, but contribute 50-75% of the total weight of endosperm starch. B-type starch granules are spherical or polygonal in shape and range from 1-10 μm in diameter. They are initiated and grown during the phase of endosperm enlargement, since about 14 days post anthesis, but their final number may be affected by environmental conditions (Parker, 1985).

Grain filling follows cell division and differentiation, when endosperm cells fill with deposits of protein and starch, which interact closely forming a starch-protein matrix, the energy-rich caryopsis component of major economic importance.

In order to evaluate the cookie quality of triticale flours, 80 lines were probed in our laboratory and 25 lines showed good performance to make cookies with higher values of cookie factor (Rubiolo *et al.*, 1992). Of these latter, the lines selected for this study were those that showed during the last five years, the highest and the lowest of both grade of shriveling and test weight values. The objective of this study was to analyze qualitative and quantitative changes of proteins and starch granules during grain filling in two triticale lines of good cookie quality. Besides, the relation of these parameters with shriveling grade of the kernel was studied.

MATERIALS AND METHODS

Samples

Two triticale experimental lines (T and B) were studied. Crops were grown in mid-level fertility soils at Campo Escuela of the Facultad de Ciencias Agropecuarias of the Universidad Nacional de Córdoba, Argentina. The lines were sowed in June 2001 with a density of 250 plants per m^2 . Each test was designed as a randomized complete block with three

replications. No watering or fertilizing was used.

Harvest was performed by hand at 14, 21, 31, 39 and 47 days after anthesis.

The Test Weight was performed according AACC method (1995)

Whole flour was obtained by milling triticale kernels on the Agromatic AG AQC 109 (Laupen, Switzerland).

The whole grains and cross sections were photographed with a Video Documentation System, Image Master VDS (Pharmacia Biotech Inc., Uppsala, Sweden)

Proteins

The protein flour content was determined by a combustion type N autoanalyzer Leco FP-2000 (Leco Corp., St Joseph, MI, USA) (Method 992.23.32.2.02., AOAC, 1998).

The proteins were studied performing a total extraction procedure and a protein sequential fractionation. Total proteins were extracted from 0.5 g of whole meal for each sample with 0.063 M tris/HCl pH 6.8, 2% SDS, 10% glycerol, with and without 5% β -Mercaptoethanol (reduced and unreduced extracts respectively) (Ng and Bushuk, 1987). Protein fractionation was performed according to a modification of the sequence used by Lupano and Añon (1985). Extraction was performed from 1 g of whole flour using three solvents: i- 10 ml of 5% NaCl for 2 h with constant agitation (albumin-globulin fraction), ii- 10 ml of 70% isopropanol for 2 h with constant agitation (prolamin fraction), iii- 10 ml of 0.063 M tris/HCl pH 6.8, 2% SDS for 2 h with constant agitation (glutelin fraction).

The protein concentration was determined in each soluble fraction (total protein, albumin-globulin, prolamin and glutelin) by micro Kjeldahl nitrogen analysis (AACC, 1995) using UDK 126 A Steam distilling unit, VELP Scientifica (Italy)

Gel electrophoresis

Electrophoresis under dissociating conditions was performed in polyacrylamide SDS gels (SDS-PAGE) according to Laemmli (1970). Total protein extracts were applied directly to the gel.

A multistacking SDS-PAGE procedure was used to determine the size distribution of polymeric proteins under unreducing conditions. Four stacking gels (pH 6.8) of 4, 6, 8 and 10% acrylamide concentration were laid on top of a 12% acrylamide resolving gel (Khan and Huckle, 1992).

Densitometry and quantification of protein bands

Gels were analyzed by densitometry in an Image Master VDS (Pharmacia Biotech Inc., Uppsala, Sweden) using the software image master VDS. A blank lane was used to obtain the background signal. The volume of protein band (integrated optical density, IOD) was represented by the following expression:

$$\text{IOD} = (\text{mean intensity} - \text{background}) \times \text{band area}$$

The proportions of protein fractions relative to total protein in the corresponding lane were quantified as IOD from each band/total IOD of the lane \times 100.

At least three determinations per point were made and average values were determined.

Starch

Starch was isolated from whole flour (300 mg) using a protein digestion procedure with pepsin and hemicellulase followed by a detergent mix treatment according to the method of Betchel and Wilson (2000). Isolated starch was resuspended in 1 ml of water and a 1:10 starch:water dilution was done before counting. Granular size distribution was determined on a Light Scattering Particle Size Analyzer Beckman Coulter LS 230 capable of measuring from 0.3 to 80 μm particle sizes.

Statistical Analysis

The samples were grown by triplicate. The data obtained were statistically evaluated by variance analysis (ANOVA). The comparison of means was done by the Tukey test at a level of 0.05. Both routines were carried out using the statistical analysis package InfoStat, statistical software (Facultad de Ciencias Agropecuarias, UNC, Argentina).

RESULTS AND DISCUSSION

Kernel weight and protein content

The kernel weight values at maturity for triticale lines T and B in 2001 were 68.80 kG/hL and 64.35 kG/hL respectively, in agreement with the high shrivelling appearance of B kernels (Figure 1). The kernel weight mean values obtained from 1998 to 2000 for T line were 69.25; 70.15; 69.75 kG/hL and for B line were 66.00; 64.35; 65.05 kG/hL. Results indicates that the lower kernel weight value and the high shrivelling appearance of B line are not due to environmental factors. Physiological maturity (PM) was reached at 47 days after anthesis (DAA) in both lines.

The percentage of total protein of maturation stages (14, 21, 31, 39 and 47 DAA) for the T line were: 11.58 ± 0.03 , 10.18 ± 0.20 , 11.92 ± 0.08 , 11.30 ± 0.12 , 11.37 ± 0.22 respectively; for the B line they were 12.52 ± 0.35 , 10.80 ± 0.47 , 10.96 ± 0.15 , 11.32

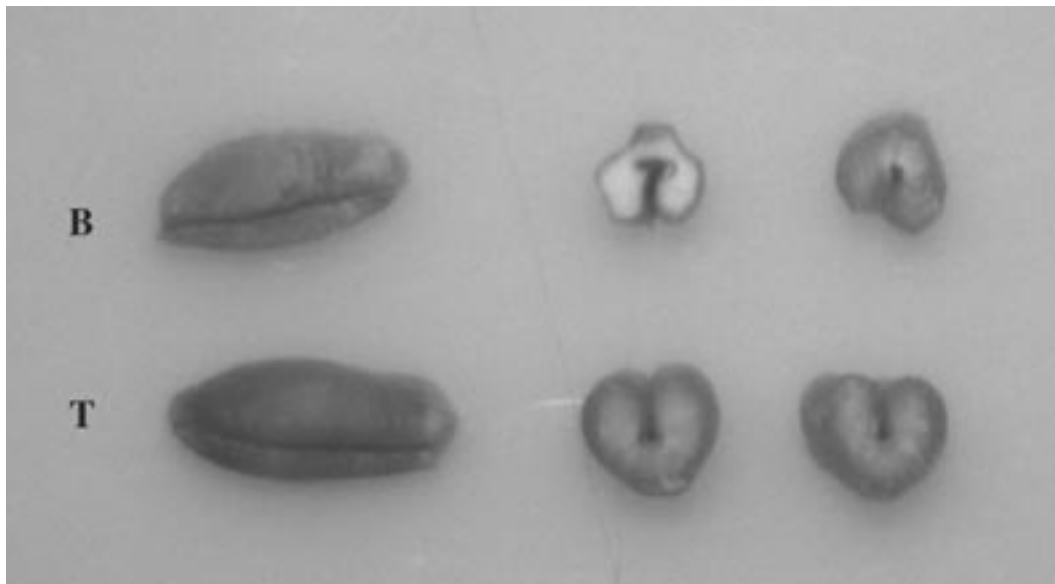
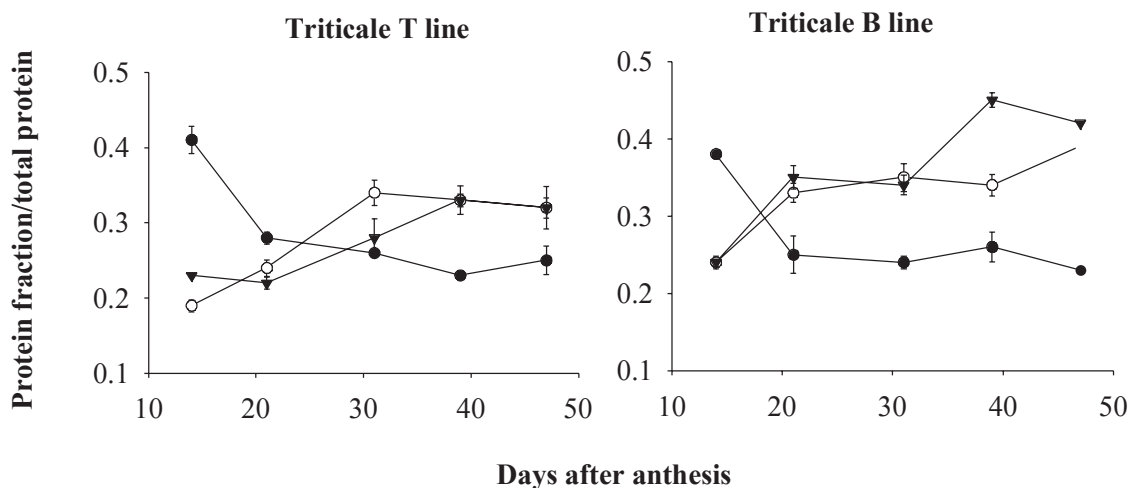


Figure 1: Front view and cross section of triticale kernels corresponding to B and T lines.

Table 1: Relative size distribution of total proteins during triticale grain filling quantified as IODr

Stage DAA	T line Mr			B line Mr		
	>60,000 (%)	59,000-40,000 (%)	< 39,000 (%)	>60,000 (%)	59,000-40,000 (%)	< 39,000 (%)
14	19.39 ± 1.3	28.21 ± 0.91	52.41 ± 1.86	19.33 ± 1.10	38.84 ± 2.27	41.83 ± 1.23
21	16.79 ± 2.7	31.07 ± 1.15	53.14 ± 1.34	19.40 ± 0.81	36.30 ± 1.55	44.30 ± 2.13
31	25.31 ± 0.9	26.10 ± 1.70	48.59 ± 1.83	23.64 ± 0.78	45.92 ± 1.37	30.44 ± 2.32
39	27.32 ± 0.6	30.33 ± 0.98	42.35 ± 1.62	35.54 ± 1.26	38.58 ± 1.59	25.87 ± 0.91
47	35.36 ± 1.7	38.97 ± 1.17	25.67 ± 1.77	37.26 ± 1.24	34.77 ± 1.44	27.97 ± 0.98

**Figure 2:** Changes in albumin-globulin (•), prolamin (o) and glutelin (▼) proportions in total proteins from developing grains of T and B triticale lines.

± 0.54, 10.82 ± 0.12 respectively. Protein content accumulation dropped at 21 DAA and increased again at 31 DAA. Probably, the high protein content at 14 DAA in both lines apparently could be explained by a difference in rate of protein and starch synthesis and the low starch content in endosperm cells at this developmental stage. In wheat, the starch content at 14 DAA was 14% and increased to 35% for 39 DAA (Betchel *et al.*, 1990). Since 21 DAA the percentage of protein was not modified. At maturity the T line showed higher protein value than the B line ($p < 0.05$).

Changes in albumin-globulin, prolamin and glutelin composition determined as percentage of total protein, during grain development are shown in Figure 2.

The proportion of protein present as albumin-globulin declined significantly throughout the stages

of grain development. These results are in agreement with data obtained in wheat (Pannoizzo *et al.*, 2001). During the early stages of grain filling the caryopsis contained predominantly albumin and globulin proteins (Kasarda *et al.*, 1976). In subsequent weeks after anthesis, there was a decrease of albumin and globulin proteins and a rapid increase in the percentage of prolamin fractions. The most rapid phase for prolamin synthesis occurred between 14 and 31 DAA. Since 31 DAA the percentage of prolamin present was not significantly different from the percentage present in the mature grain (Figure 2).

In the T line, glutelin proteins were initially synthesized at a slower rate than prolamins, but in line B both protein types were synthesized at the same velocity.

Glutelin protein content increased significantly

since 14 DAA ($p < 0.05$) in B and T lines. Between 21 and 39 DAA, there was a rapid increase in glutelin synthesis and it remained constant after 39 DAA (Figure 2). Previous studies performed in wheat (Khan and Bushuk, 1976; Kaczkowski *et al.*, 1987) have shown that glutenin existed in lower proportion than albumin and globulin at early developmental stages, and after that, glutenin fraction increased at different rates until full maturity. The protein synthesis pattern was characterized by the accumulation of one protein type and the decrease of another. The decrease in albumin and globulin protein coincided with the rapid phase of glutelin synthesis, which suggests that the non-prolamine proteins may be precursors of the synthesis of larger storage proteins at the end of grain filling period. In support of this, Gupta *et al.* (1991) and Field *et al.* (1983) reported that non-prolamin protein polymerize through disulfide interchange reactions to form polymeric glutenin in wheat.

Electrophoresis

SDS-PAGE results indicated that after 14 DAA and up to the maturity stage, the patterns of total protein were essentially the same except for the variation in band intensities. The densitometric analysis of electrophoretic patterns of proteins extracted at different grain filling periods is shown in Table 1. Throughout grain filling in both triticale lines, the relative IOD increased for proteins of Mr higher than 60,000 and decreased for proteins lower than 39,000, although T line had higher proportion of small proteins from 14 to 39 DAA than B line. Conversely, the intermediate size proteins reached the highest proportion at 31 DPA in B line and at 47 DPA in T line. The ratio large/medium proteins increased throughout grain development arriving at their stable proportion at 31 DAA in T line and at maturity in B line (Table 1). These results showed that proteins of dif-

Table 2: Percentage volume of starch granules in T and B triticale lines during grain filling

Stage DAA	% Volume			
	T line		B line	
	Small granules	Large granules	Small granules	Large granules
14	18.71 ^a	81.29	19.19 ^a	80.81
21	26.94 ^b	73.06	38.07 ^{c, d}	61.93
31	32.61 ^{b, c}	66.39	42.58 ^{d, e}	57.42
39	34.20 ^c	65.80	45.14 ^e	54.86
47	36.54 ^{c, d}	63.46	45.40 ^e	54.60

large granules = A type, small granules = B type

Values followed by the same letter are not significantly different ($p < 0.05$)

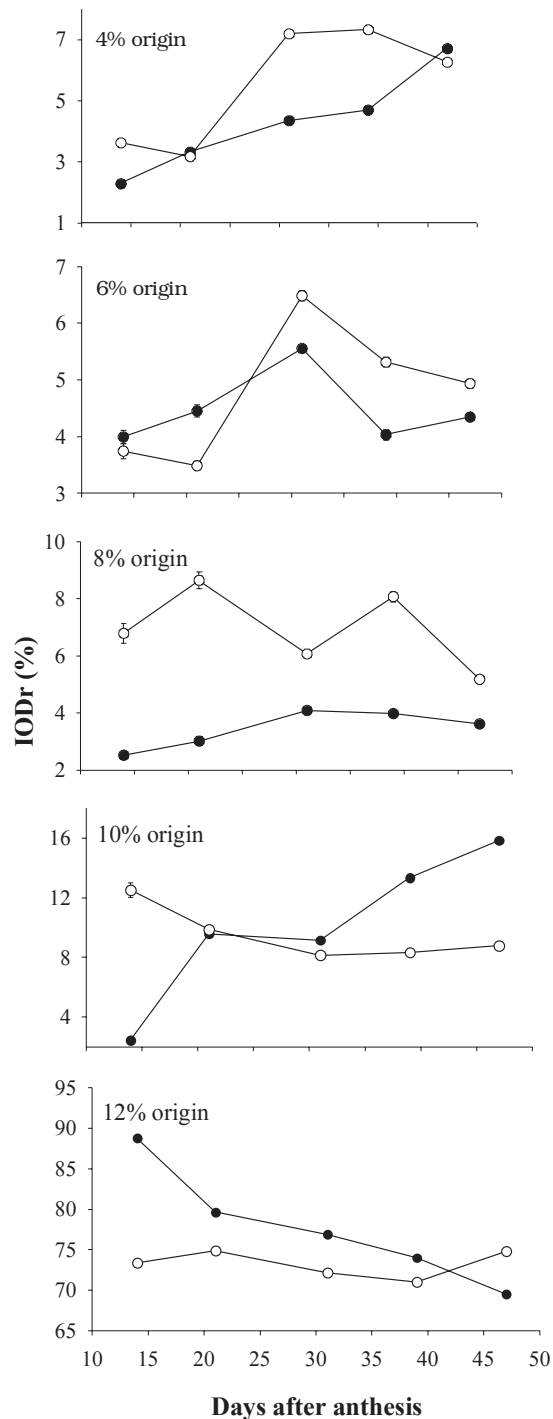


Figure 3: Changes of polymerization grade during triticale grain development quantified as IODr from multistacking SDS-PAGE patterns. (•) T line; (○) B line.

ferent molecular weight differed between both lines in the timing of their deposition. The differences in proportional size distribution at each developmental stage could be an influencing factor on the shrivelled appearance of the B line.

Unreduced SDS-soluble protein aggregates were separated by multistacking polyacrylamide gel to investigate the size distribution of native protein. The molecular size of aggregates increased throughout grain filling. The proportion of large size polymers retained at 4% acrylamide concentration stacking gel increased significantly from 21 DAA (Figure 3) as it has also been reported in wheat by Zhu and Khan (1999). These polymeric aggregates probably are synthesized from large subunits (higher than 60,000) that increased their synthesis at latter stages of grain development (Table 1). The polymerization process is most likely via disulfide interchange reactions, although the mechanism of how the different aggregates polymerize is not known (Panozzo *et al.*, 2001).

In the T line, the percentage of protein aggregates accumulated in 10% stacking gel increase during the grain filling (2,43 to 15,83%), while in B line, it decreased throughout grain development (12.51 to 8.77%) (Figure 3).

The percentage of proteins separated in a 12% resolution gel remained constant in the B line during grain development otherwise in the T line it decreased 20% between 14 and 47 DAA (Figure 3). Smaller polymers and protein subunits separated in 12% resolution gel are the most likely precursors of the formation of larger polymers and they decreased as larger polymers increased. These results suggest that T and B lines had differences in the formation of gluten matrix during grain development.

Table 3: Particle size of the highest frequency in each stage of grain development

Stage DAA	Particle Size (μm)			
	T line		B line	
	Small granules	Large granules	Small granules	Large granules
14	2.54	18.86	3.21	17.18
21	9.82	22.73	9.82	20.70
31	9.82	22.73	5.11	22.73
39	9.82	22.73	5.11	20.70
47	5.11	22.73	5.11	22.73

Starch

Size distribution of the starch granules from two triticale lines during grain filling are shown in Figure 4. The diagrams illustrate the appearance of the two main fractions of starch granules, small (B-type) and large (A-type). The data revealed a bimodal distribution of granules in triticale endosperm from 14 DAA until maturity. The limit between the two fractions was defined as the minimum of the curve. Granules smaller than the limit are called small granules while granules with larger diameters are called large granules. It is widely acknowledged that, at maturity, wheat contains two types of starch granules: larger and smaller than 10 μm . In the early stage of grain development (14 DAA) of both triticale lines the small granules were smaller than 5 μm , while since 21 DAA, they increase in size up to 10 μm (Figure 3). Bimodal behavior was in agreement with Karlsson *et al.* (1987). Other studies in wheat have also reported two classes of starch granules (Brocklehurst and Evers, 1977; Baruch *et al.*, 1983; Karlsson *et al.*,

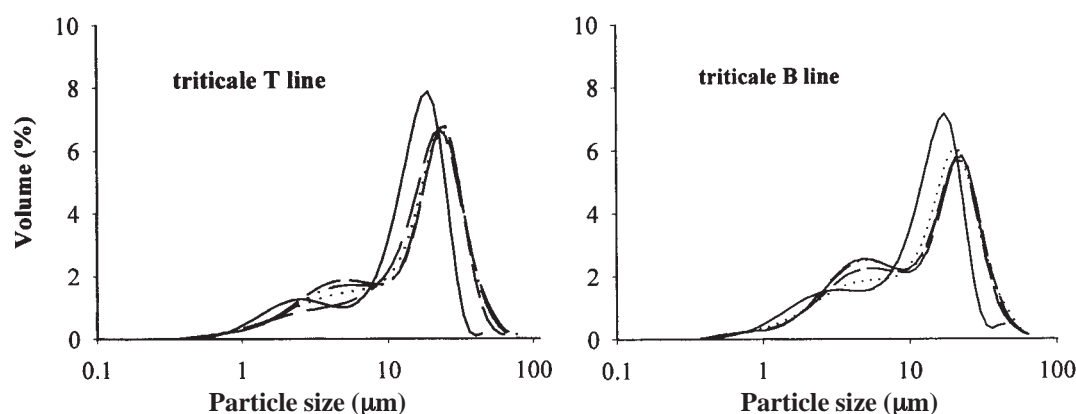


Figure 4: Size distribution of starch granules in T and B triticale lines during the grain filling period. 14 DAA (—), 21 DAA (---), 31 DAA (....), 39 DAA(— · —) and 47 DAA (----)

1983; Morrison and Scott, 1986), however, Betchel *et al.* (1990) and Raecker *et al.* (1998) reported three classes of wheat starch granules (A, B and C) because they could detect granules smaller than 3 μm . In the present study only two types of starch granules were identified although a light scattering particle size analyzer capable of measuring from 0.3 μm was used.

The percentage volume of large granules was reduced during grain filling while the proportion of small granules was increased (Table 2) in agreement with Karlsson *et al.* (1987). The synthesis of A type granule begins in amyloplasts four days after flowering (Buttrose, 1963; Parker, 1985; Betchel *et al.*, 1990). The synthesis of B type granules begins at 12 – 14 DAA and increases in size up to 10 μm (Betchel *et al.*, 1990). The number of B-type granules increases throughout most stages of grain development, whereas the number of A-type granules remains constant while the size increases up to about 50 μm (Parker, 1985; Morrison and Gadan, 1987).

T line differs from B line especially in the content of small granules. T line presented from 21 DAA to maturity, lower proportion of small granules than B line (Table 2). Apparently since 31 DAA for both lines, the relative proportion of small granules did not change significantly throughout grain development.

Table 3 shows the particle size that presented the highest frequency for small and large granules. In the T line the maximum of the distribution curve changed between 14 and 21 DAA and remained constant up to 39 DAA while in the B line small granules had a different pattern of distribution and size frequency. Particle size of small granules decreased significantly from 31 DAA. At maturity both lines presented the same diameter frequencies.

The highest frequency of smaller size particles (Table 3) and the greater percentage of volume of small amyloplast (Table 2) found in the B line could be associated with the high level of shrivelled grain observed in this line. However, Karlsson *et al.* (1987) observed a low percentage of small granules in a shrivelled genotype of triticale, but it showed a smaller mean diameter of granules according to our results. It is not clear if there is any connection between the relative proportion of small/large granules and the shrivelling of the kernel on account of the influence of the environmental conditions (temperature and humidity) on the small/large granule relation (Shi *et al.*, 1994).

The failure of endosperm cells to completely fill their grain sinks is the phenomenon most likely responsible for the grain shrivelling in triticale. Deposits of protein and starch interact intimately

forming a starch-protein matrix that fills endosperm cells. The differences of deposition rate of prolamin and glutelin (Figure 2), and in the proportion of larger polymers (Table 1) observed in both lines and the different size distribution and size frequency of the starch granules during the grain filling would affect the starch-protein interaction and, consequently, the higher shrivelled appearances of the B line.

CONCLUSION

In triticale, the synthesis of the albumin-globulin fraction decreased during grain filling while prolamin and glutelin synthesis increased. Protein content and nonreducing multistacking gel electrophoresis showed differences in protein synthesis between T and B triticale lines. The most important differences between the two lines were the synthesis rate of that prolamin and the amount of the large protein polymers at the end of the grain filling.

Starch was present in a bimodal size distribution in both triticale lines. Proportion of the large granule decreased while proportion of the small granule increased during ripening. Differences in granule size, percentage volume of granules and the way of that proteins are being accumulated would be related to shriveling level of grains.

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