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Effect of N fertilization on storage protein and some amino acid quantities of two Tunisian Barley varieties differing in their degrees of adaptation to environmental conditions

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Résumé

Deux variétés tunisiennes d'orge (Martin et Rihane) différant de point de vue adaptation aux conditions environnementales ont été cultivées sous différents niveaux de fertilisation azotée. L'augmentation de l'apport d'azote (N) induit une élévation de la quantité d'azote dans les grains et une diminution du poids du grain et de l'albumen. L'effet de la fertilisation azotée sur les constituants des protéines de réserve (exprimées en mg/albumen) a été déterminé par électrophorèse (Nu-PAGE) utilisant le tampon de migration (MOBS) associée à une analyse densitométrique. L'effet de l'apport azoté sur la variation de la fraction des hordéines (B) et sur quelques acides aminés (AA), particulièrement la cystéine impliquée dans les ponts disulfure des agrégats protéiques, a été discuté en relation avec le degré d'adaptation aux conditions environnementales qui caractérisent chaque variété.

Mots clés: *Orge, B-Hordéines, cystéine, adaptabilité, fertilisation azotée.*

Abstract

Two Tunisian barley varieties, Martin and Rihane, differing in degrees of adaptation to environmental conditions, were grown under different treatments of nitrogen (N) fertilization. Total nitrogen content increased when intensifying N supply whereas grain and albumen weights decreased. Effect of N fertilization on storage protein components (expressed in mg per albumen) was determined by Nu-PAGE electrophoresis system using the MOBS running buffer and densitometry analysis. The effect of N supply on the change of B-hordein fraction and some amino acids (AA) particularly the cysteine, implicates in disulfide bonds of protein aggregates, was discussed in relation to different degrees of adaptability to environmental conditions which characterize each barley variety.

Keywords: *Barley- B-hordeins- cysteine- adaptability-N-fertilization.*

ملخص

ملخص تاثير التسميد الازوتي على نسبة مخزون البروتينات و بعض الحوامض الامينية عند صنفين من الشعير التونسي متباينين من ناحية التأقلم مع الظروف البيئة. لقد تمت زراعة صنفين من الشعير التونسي (مرتان و ريحان) متباينين من ناحية التأقلم تحت كميات مختلفة من التسميد الازوتي. و لقد تبين أن الزيادة في التسميد يولد زيادة في كميات الازوت في الحبة و نقصا في وزنها و وزن مخزونها. و لقد وقع تقييم تأثير التسميد الازوتي على نسبة المكونات الهر دينية لحب الشعير بواسطة الفرز بالرحلان الكهربائي (الاستشراد) و استعمال هلام اكر يل الاميد و محلول الطابع و ذلك باعتماد تحليل الكثافة السمكية. و لقد تمت مناقشة تأثير الاستعمال المكثف للازوت على نسبة الهر دينيات "ب" و بعض الحوامض الامينية و خاصة السستيين التي تلعب دورا في تجميع البروتينات بواسطة جسور السلفير استنادا إلى درجة تأقلم الصنفين من الشعير مع الظروف البيئة

الكلمات المفتاحية: *شعير الهر دينيات "ب" سستيين تأقلم .*

Barley is an important cereal crop in Tunisia where it is used mainly as a grain for human and animal nutrition. Breeding of new barley varieties mainly focuses on high grain yield, which has led to new varieties like Rihane being introduced. A high level of grain production and a greater influence of weather and site conditions characterize this variety. However, old varieties, like Martin, are still grown because they easily adapt to stress especially in marginal unpredictable environmental. Barley varieties Martin and Rihane were considered because they are the only two breeding varieties cultivated in Tunisia, possessing a high grain yield (Daâloul, 1996). However, grain quality depends on the alleles expressed, the total amount of proteins and the balance between the different protein groups. This balance and the level of protein expression can be modulated by increasing N fertilization (Martin et al., 1992; Fatta et al. 2000). In barley grain, the major N sink is represented by storage proteins, which are soluble in concentrated alcohol solutions and are called “hordeins” (Giese et al., 1983; Autran et al., 2001). However, hordeins can be divided into four groups A, B, C and D. The B- and C-hordeins are the main storage protein groups (Shewry and Miflin, 1982). According to Shewry et al. (1994), they belong respectively to sulfur-rich and sulfur-poor prolamin families and are also homologous respectively to low molecular weight glutenins and to ω-gliadins of wheat. Like low molecular weight-glutenins, B-hordeins form aggregates stabilized by intra and intermolecular disulfide bonds (Brandt et al., 1985). Consequently, the aim of this work was to determine the quantitative variations of storage protein fractions A-, B-, C-, D-hordeins extracted from albumens of the two barley varieties Martin and Rihane grown under increasing N levels. A particular attention was accorded to assess the effect of N supply on the change of B-hordein fraction and some amino acids particularly the cysteine, implicates in disulfide bonds of protein aggregates, in relation to different degrees of adaptability which characterize each barley variety to environmental conditions.

MATERIALS AND METHODS

The Genetic Laboratory of the National Agronomic Research Institute of Tunisia provided the barley grains of the Martin and Rihane varieties. These two varieties were grown in two plots at the experimental station of Cherfech, 30 km northwest of Tunis. Rates 0, 40, 80, 120 kg/ha^{-1} of N fertilizer (ammonium nitrate 33.5% of N) per cycle of growth were applied on four different dates (early seeding stage, tillering, stem elongation and ear emergence stages respectively) according to the following pattern: 0 (0,0,0,0); 40 (20,20,0,0); 80 (20,20,20,20); 120 (40,40,20,20).

Fifteen random samples per treatment (5 replications x 3 repetitions) were mixed to produce about 1.5 kg of seeds. Sampling of the mixed seeds was taken using a laboratory divider to obtain homogeneous samples of about thousand grains. Ten grams were milled for Kjeldahl protein determination (P =5,75 x N). Albumen was isolated according to Eynard and Laurière (1998) and milled just prior to use. Whole meal of albumen (25mg) was extracted in 2ml Eppendorf tubes, with Laemmli sample buffer (pH=8.5) containing 5% 2-mercaptoethanol (Laemmli, 1970).

Electrophoretic analysis of total albumen proteins was done on ready-made Novex 4-12% Bis-Tris-HCl polyacrylamid gradient gels (Introgen CA USA) at 50 mA constant current per slab gel for 1 h 15 mn, using the recommended MOBS [3-(N-morpholino) propane sulfonic acid] running buffers pH 7,7. Images of stained gels were numerated on a normal office scanner and analyzed using the Wilbert-Lourmat Bio 1D software (France), taking into account both the whole surface and the intensity of each protein band.

The amino acids analysis was realized by phenylthiocarbamyl derivatives methods as described by Bidlingmeyer et al. (1984).

RESULTS AND DISCUSSION

Nitrogen content, albumen and grain weight

Increasing N supply from 0 to 120 kg ha^{-1} increased the nitrogen content of maturing grain of Martin by about 27 % but that of Rihane increased only by about 12% (Table 1). Table 1 also shows that N fertilization caused a decrease in grain and consequently in albumen weights, in both varieties, but the decrease was more important in Martin in relation to its high nitrogen content. Similar results were obtained by Bruckner and Morey (1988) indicating a negative relation between the two cited parameters when intensifying N supply at early stages of growth (N treatment 120 kg ha^{-1}). The influence of timing and rate of N fertilization on quality and yield of cereals particularly wheat varieties was well documented (Roth and Marshall, 1987; Ayoub et al., 1994). Contradictory results found could be attributed to differences of environmental conditions particularly climatic conditions (Zebarth and heard, 1992). So, it has been suggested that partitioning N supply with multiple applications will minimize the effect of the environment, particularly when the greater part of N supply is applied at early stages of plant development as be employed in the present work (Gravelle et al., 1988), by improving the efficiency of nitrogen use (Mullen et al., 2003).

Table 1: Nitrogen content (% dry matter), grain and albumen weights of Martin and Rihane as influenced by N supply. Values are the averages (± standard deviation) of 3 repetitions per treatment (values of each repetition is the average of 5 replications) at 0.05 significance levels.

N levels (kg/ha)	Nitrogen content	Grain weight		Albumen weight	
	N (% DM)	Dry weight (mg)	Humidity (%)	Dry weight (mg)	Humidity (%)
		Variety Martin			
0	1,75±0,09	40,40		33,71±1,42	
40	1,84±0,08	±1,48	7,34	32,40±1,13	8,99
80	1,93±0,04	39,60±1,26	7,26	31,14±1,28	8,38
120	2,23±0,12	38,80	6,95	28,99±1,19	8,26
		±2,27	7,94		8,55
		34,80±2,08			
		Variety Rihane			
0	1,72±0,05	38,10±1,52	7,52	31,01±1,23	8,43
40	1,83±0,09	36,40±1,01	7,61	30,23±0,88	8,40
80	1,87±0,07	36,20±1,33	7,18	30,01±1,39	8,51
120	1,93±0,10	35,40±1,21	7,09	29,54±1,06	7,87

Nu-PAGE electrophoresis

As be shown by Figure 1, the major storage proteins called “Hordeins” were separated by electrophoresis Nu-PAGE MOBS into four fractions. The B- and C-hordein polypeptides represented the main fractions and were ranged respectively from 30 to 45 kd and from 45 to 80 kd in both varieties. The C-hordein patterns comprised three bands in Rihane (R) and four bands in Martin (M). Analysis of the B-hordeins of Martin showed that this protein fraction comprised five polypeptidic bands, while those of Rihane comprised three. Thus, it appears that Martin presents a high degree of polymorphism of B- and C-hordein electrophoresis patterns compared to Rihane. In previous work, the high polymorphism of B- and C-hordeins was found with a high adaptability to environmental conditions of traditional Tunisian barley cultivars (Bettaieb-Ben kaab and Attias, 1992).

Effect of N fertilization on hordein fractions

The major B-hordeins fraction was differently affected by increasing N fertilization in the two varieties (Figure 2).

The A-hordein polypeptides scarcely increased in the two varieties, whereas the minor D-hordein fraction decreased in Martin and increased in Rihane (figure 2). The accumulation of the C-hordein fraction increased with

increasing N supply in both varieties mainly at the highest levels of N fertilization (Figure 2). Obviously, the C-hordein polypeptides seemed, in the present work, to act as the main nitrogen sink at the highest levels of N supply, which is in agreement with previous study described in litterature (Giese et al., 1983, Wieser and Seilmeier, 1998, Daniel and Triboi, 2000). So, it is important to observe that results obtained on B-hordein fractions of the variety Rihane contrasted with those of the variety Martin, in which the B-hordein fraction did not response to N fertilization (Figure 2). They suggested that the constancy of the B-hordein quantities of the old variety.

Martin under all N treatments will be probably at the origin of its high adaptation to stress conditions. But these findings will be confirmed on many traditional cereal varieties or cultivars especially barley which easily adapt to difficult environmental conditions as did the old variety Martin.

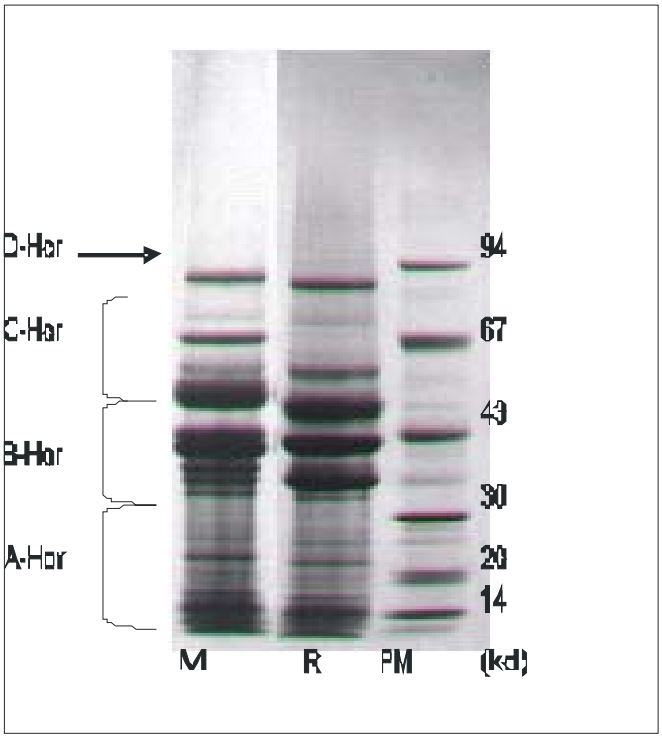

Figure 1: Electrophoresis Nu-PAGE patterns of the hordein components D-, C-, B- and A- hordeins (Hor) extracted from albumen of two barley varieties Martin (M) and Rihane (R). Proteins were reduced with antioxidant prior electrophoresis. The buffer gel is Bis-Tris- HCl and the running buffers are the MOBS. Protein Markers (PM) were Phosphorylase (94 kd), Bovine Serum Albumine (67 kd), Ovalbumine (43 kd), Anydrase Carbonique (30 kd), Trypsine I of Soya (20 kd) and α-Lactoglobuline (14 kd).

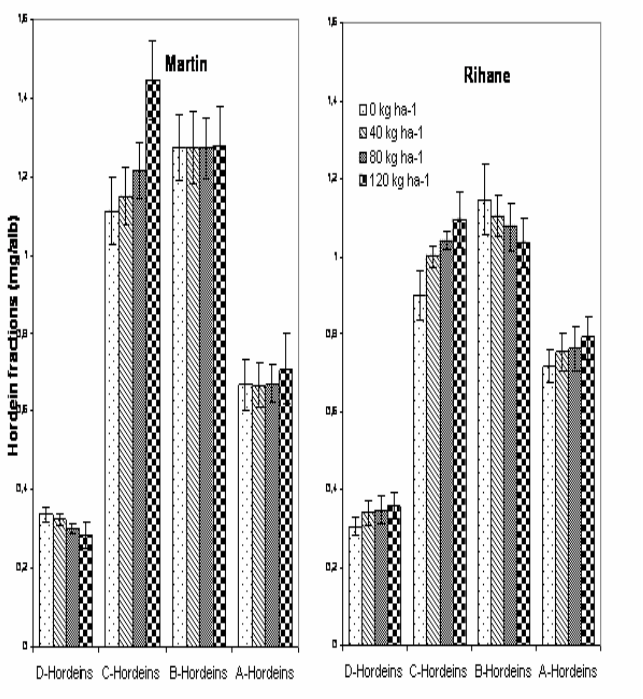

Figure 2: Amount changes of different hordein fractions D-, C-, B- and A-Hordeins expressed in mg per albumen and separated by Nu-PAGE MOBS of barley varieties Martin and Rihane in relation to increasing levels of N fertilization, values are the averages (± standard deviation) of 5 repetitions per treatment at 0,05 significance levels.

Amino acid analysis

The AA composition of Martin and Rihane grown under varying levels of N fertilizer was given respectively in Tables 2 and 3, and was expressed in µg AA/grain and in percentage of total AA.

As be illustrated by Tables 2 and 3, results show a great variability of most grain amino acids of the two barley varieties grown under increasing N fertilization. Variations of AA content were more pronounced in Martin than in Rihane. However, the amounts of Glx and proline, expressed in µg per grain, was much more affected by N supply in Martin than in Rihane and revealed a varietal-dependent increase of C-hordeins which are Glx-rich and proline-rich according to Shewry et al, (1994). The lysine concentration, expressed in percentage, decreased with increasing N levels of fertilizer in both varieties, but more markedly in Martin than in Rihane. Its grain rate registered a decrease by about 18% in Martin and by about 7% in Rihane (Table 2 and 3).

In addition, N supply caused an increase of the amount of the cysteine (µg/grain) by about 35%, but didn't affect its percentage in the variety Martin (Table 2). In the opposite side, in the variety Rihane, the cysteine amount and percentage decreased with increasing N supply by about 16 % (Table 3). In the same way, many studies have shown that environmental factors, mainly N fertilization act on polypeptides sulfur-rich subunits, possessing multiple

cysteine residues, represented in wheat by LMW-glutenins and in barley by B-hordeins, and will modified the molecular weight and size and the distribution of polymers which are stabilized with inter-chain disulfides bonds S-S and are involved in protein aggregates (Scheromm et al., 1992; Jia et al., 1996a; 1996b).

However, polymeric proteins could be different among cultivars. The differences might exist in B-hordein proteins polymerization, which would contribute to the differences in the genetic quality of response to environmental conditions.

Table 2: amino acid (AA) analysis of maturing grain of the variety Martin expressed in µg per grain and in percentage, as influenced by N fertilization.

N Levels (kg ha^{-1})	N_0		N_{40}		N_{80}		N_{120}	
AA	µg/grain	AA(%)	µg/grain	AA(%)	µg/grain	AA(%)	µg/grain	AA(%)
Asx	235,5	5,87	241,5	5,71	274,9	6,10	333,5	6,14
Glx	1042,8	25,98	1089,0	25,76	1189,7	26,40	1469,9	27,08
Ser	175,3	4,36	173,2	4,10	212,8	4,72	238.8	4,40
Gly	176,6	4,40	203,6	4,82	182,9	4,06	207.9	3,83
His	101,1	2,52	105,7	2,50	95,1	2,11	118.8	2,19
Arg	184,1	4,59	182,7	4,32	218,7	4,85	267,6	4,93
Ala	168,7	4,20	174,5	4,13	193,8	4,30	222,0	4,09
Pro	366,5	9,13	448,1	10,60	521,2	11,57	653,0	12,03
Thr	141,1	3,51	145,3	3,44	167,1	3,71	191,6	3,53
Tyr	141,5	3,52	147,5	3,49	138,1	3,07	163,4	3,01
Val	223,7	5,57	234,0	5,54	225,5	5,00	282,2	5,20
Met	70,3	1,75	63,1	1,49	85,1	1,89	104,7	1,93
Cys	118,6	2,96	127,9	3,03	129,9	2,88	160,2	2,95
Ile	168,3	4,19	174,0	4,12	161,6	3,59	206,8	3,81
Leu	266,7	6,65	281,9	6,67	273,1	6,06	322,6	5,94
Phe	231,6	5,77	224,9	5,32	227,3	5,05	260,2	4,79
Lys	141,5	3,53	150,9	3,57	145,4	3,23	156,0	2,88
Trp	60,2	1,50	59,2	1,40	63,4	1,41	68,6	1,26
Total	4014,1	100,00	4227,0	100,01	4505,6	100,00	5427,8	99,99

Table 3: Amino acid (AA) analysis of maturing grain of the variety Rihane expressed in µg per grain and in percentage, as influenced by N fertilization.

N levels (kg ha^{-1})	N_0		N_{40}		N_{80}		N_{120}	
AA	µg/grain	AA (%)	µg/grain	AA (%)	µg/grain	AA (%)	µg/grain	AA (%)
Asx	290,2	6,67	270,1	6,53	267,3	6,44	235,3	5,55
Glx	1064,0	24,46	1049,7	25,36	1021,1	24,59	1066,7	25,17
Ser	205,5	4,72	197,0	4,76	202,2	4,87	201,7	4,76
Gly	178,6	4,11	170,4	4,12	168,6	4,06	181,0	4,27
His	96,9	2,23	86,0	2,08	91,4	2,20	106,4	2,51
Arg	231,6	5,32	208,6	5,04	220,4	5,31	196,5	4,64
Ala	195,0	4,48	187,4	4,53	181,9	4,38	182,3	4,30
Pro	458,3	10,54	457,2	11,05	455,3	10,97	473,6	11,18
Thr	154,6	3,55	147,1	3,56	149,2	3,59	141,3	3,34
Tyr	133,9	3,08	125,5	3,03	127,4	3,07	149,1	3,52
Val	231,6	5,32	223,2	5,39	222,8	5,37	236,2	5,58
Met	91,0	2,09	84,8	2,05	77,6	1,87	80,1	1,89
Cys	156,7	3,60	139,2	3,36	135,9	3,27	131,4	3,10
Ile	169,7	3,90	144,6	3,49	165,8	3,99	168,5	3,98
Leu	270,4	6,22	262,7	6,35	257,6	6,20	273,7	6,46
Phe	200,5	4,61	189,9	4,59	203,8	4,91	209,0	4,93
Lys	161,3	3,71	136,7	3,30	147,2	3,54	146,5	3,46
Trp	60,6	1,39	58,6	1,42	57,01	1,37	58,2	1,37
Total	4350,4	100,00	4138,7	100.01	4152,5	100,00	4237,5	100,01

The variation of cysteine quantities, different between the two varieties, when intensifying N supply will revealed differences in sulfur metabolism and in molecular weight and size of B-hordein aggregates and will explain differences in behaviors between the two barley varieties in the degree to which they can adapt to various environmental conditions. Although, these results have to be extended over a large number of barley cultivars and will provide a basis for further investigations of association between B-hordein subfractions with different sizes and molecular weights, sulfur metabolism and the quality of response to changes of environmental conditions, particularly N fertilization.

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REFERENCES

[1]- Autran, J.C., Halford, N., Shewry, P.R.,. 2001. Nitrogen and seed storage proteins: The biochemistry and molecular biology of seed storage proteins. In: The nitrogen assimilation by plants (J.F. Morot-Gaudry and P. Lea, eds.) Springer. Verlag, 295-341.

[2]- Ayoub, M., Guertin, S., Lussier, S., 1994. Timing and level of nitrogen fertility. Effects on spring wheat yield in eastern Canada. Crop. Sci. **34**: 748-756.

[3]- Bettaieb-Ben Kaâb, L., Attias, J., 1992. Electrophoresis heterogeneity of the hordein five barley (*Hordeum vulgare* L.) cultivars from Tunisia. C.R. Acad. Sci. Paris, Serie III. **314**: 141-146.

[4]- Bindlingmeyer, B.A., Cohen, S.A., Tarvin, T.L., 1984. Rapid analysis of amino acids using pre-column derivatization. J.Chromatogr. **336**: 93-104.

[5]- Brandt, A., Montembault, A., Cameron-Mill, V., Rasmussen, S.K., 1985. Primary structure of a B1 hordein gene from barley. Carlsberg. Res. Commun. **50**: 333-345.

[6]- Brukner, P.L., Morey D.D., 1988. Nitrogen effects on soft red winter wheat yield, agronomic characteristics and quality. Crop. Sci. **23**: 152-157.

[7]- Daâloul A., 1996. Les resources phytogénétiques des cereales en Tunisie. Séminaire sur la conservation et la valorisation des ressources génétiques des plantes du Maghreb. Projet FEM/PNUD/FAO, 12-14 Mars 1996. Sidi Thabet, Tunisie.

[**8]-** Daniel, C., Triboi, E., 2000. Effects of temperature and nitrogen nutrition on the grain composition of winter wheat: effects on gliadin content and composition . J. Cereal. Sci. **32**: 45-56.

[9]- Eynard L., Laurière M., 1988. The combination of Indian ink staining with immuno- chemiluminescence detection allows precise identification of antigens on blots. Application to the study of glycosylated barley storage proteins. Electrophoresis.**19**: 1394-1396.

[10]- Fatta N., Caputo, C., Barneix, A.J., 2000. The absence of the short arm of chromosome 7B Produces inhibition of N mobilization and decreases grain protein concentration in wheat (*Triticum aestivum* L.) cv. Chinese Sp. Agronomie. **20**: 423-430.

[11]- Giese, H., Andersen, B., Doll, H., 1983. Synthesis of major storage protein, hordein, in barley. Planta. 159: 60-65.

[12]- Gravelle, W.D., Alley, M.M., Brann, D.E., Joseph, K.D.S.M., 1988. Split nitrogen application effects on yield, lodging and nutrient uptake of soft red winter wheat. J. Prod. Agric.1: 249- 256.

[13]- Jia, Y.Q., Fabre, J.L., Aussenac, T., 1996a. Effects of growing location on response of protein polymerization to increase nitrogen fertilization for common wheat cultivars season: Relationship with some aspects of the bread making quality. Cereal. Chem. **73**: 526-532.

[14]- Jia, Y.Q., Masbou, V., Aussenac T., Fabre J., Debaeke, P., 1996b. Effects of nitrogen fertilization and maturation conditions on protein aggregates and the bread making quality of season, a common wheat cultivars. Cereal. Chem. **73**: 123-130.

[15]- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the heads of bacteriophage T4. Nature (London). **227**: 680-685.

[16]- Martin, B.J., Sutton, K.H., Moyle T.H., Hay, R.L., Gillespie, R.N., 1992. Effect of nitrogen Fertilizer on the yield and quality of six cultivars of autumn-sown wheat. N.Z.J. Crop. Hortic. Sci. **20**: 273-282.

[17]- Mullen, R.W., Freeman, K.W., Raun, W.R., Johnson, G.V., Stone, M.L., Solie, J.B., 2003. Identifying an In-season response index and the potential to increase wheat yield with nitrogen. Agronomy J. **95**: 347-351.

[18]- Roth, G.W., Marshall, H.G., 1987. Effects of timing of nitrogen fertilization and a fungicide on softres winter wheat. Agron J. **79**: 197-200.

[19]- Scheromm, P., Martin, G., Bergoin, A., Autran, J.C., 1992. Influence of nitrogen fertilization on the potential bread-baking quality of two wheat cultivars

differing in their response to increasing nitrogen supplies. Cereal. Chem. **69**: 664-670.

[20]- Shewry, P.R., Miflin, B.J., 1982. Gene for storage proteins of barley. Plant Foods For Human Nutrition. **31**: 251-267.

[21]- Shewry, P.R., Miles, M. J., Tatham, A.S., 1994. The prolamin storage proteins of wheat and related cereals. Prog. Biophys. Molec. Biol. **61**: 37-59.

[22]- Wieser, H., Seilmeier, W., 1998. The influence en fertilization on quantities and proportions of different protein types in wheat flour. J. Sci. Food. Agric. **76**: 49-55.

[23]- Zebarth, B.J., Sheard, R.W., 1992. The influence of rate and timing of nitrogen fertilization on yield and quality of hard red winter wheat in Ontario. Can. J. Plant. Sci. **72**:13-19.