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Wheat yield and grain protein response to nitrogen amount and timing

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Abstract

This research was carried out to study the effect of nitrogen rate application (0, 120, 240 and 360 kg Nitrogen ha^{-1}) and nitrogen timing on grain yield, yield components, grain quality and protein banding pattern in different growth stages of wheat (Shiraz cultivar) which was grown at research station of the School of Agriculture, Shiraz University at Bajgah in the 2008-2009. Results indicate that the highest value for grain yield was obtained at 240 kg N ha^{-1} when it was applied through vegetative growth stages (8230 kg ha^{-1}). Yield components were significantly increased with enhancing the level of nitrogen with no significant difference between 240 and 360 kg N ha^{-1}. Results show that no N application at tillering stage decreased spikes number/m^2 and seeds $spike^{-1}$, and the minimum value of 1000 grain weight was observed when we remove N application in grain filling period. Leaf protein did not influenced by N treatments. However, the sharpness of a band with molecular weigh about 51 kDa, related to Rubisco enzyme, was increased in some treatments. Furthermore, 240 kg N ha^{-1} application in all timing treatments resulted in maximum seed protein content (5.5 mg protein g^{-1} grain). At the same time, seed water soluble proteins, Albumin and Globulin, showed no polymorphism. While different N rate treatments had no significant effect on seed gluten content, no N application in grain filling period declined it significantly. All in all, obtaining high wheat grain yield beside a suitable bakery quality are possible in a proper farming management system without environmental impact of N over application. These goals may be achieved with sufficient N application during vegetative growth for yield and late season N application for protein quality.

Keyword: Gluten, Grain yield, N fertilization, Protein, *Triticum aestivum*.

Introduction

Wheat (*Triticum aestivum* L.) is the most widely grown crop in the word with its unique protein characteristics that serves as an important source of food and energy in Iran (Cooke and Law, 1998). Mature wheat grains contain 8–20% protein, which are divided into two major categories: prolamins including gliadins and glutenins and non-prolamins consisting of water-soluble albumins and salt-soluble globulins (Singh and Skerritt, 2001). Grain quality is a complex trait resulting from the interactions between numerous protein components (Daniel and Triboi, 2000). The protein composition of wheat seeds is important in determining bread-making quality (Johansson et al., 2001). Gluten proteins, a large complex composed mainly of glutenins and gliadins, play a key role in baking quality because of their impact on water absorption capacity of the dough, dough elasticity and extensibility that can affect wheat flour quality (Torbica et al., 2007). Albumins and globulins probably do not have a critical role in flour quality, while they may have dual roles as nutrient reserves for the germinating embryo (Gupta et al., 1991) and as inhibitors of insects and fungal pathogens prior to germination besides their influence on grain hardness in some cereal (Morris, 2002). The protein content in the wheat grain is dependent on genotype but it is also clearly influenced by environmental variables such as nitrogen application, water access and temperature during growth especially through the grain filling period (Daniel and Triboi et al*.,* 2000; Luo et al., 2000; Ottman et al., 2000; Rharrabti et al., 2001; Altenbach, et al., 2002; Dupont and Altenbach 2003; Tea et al., 2004). These factors influence the rate and duration of wheat grain development, protein accumulation and starch deposition (Jamieson et al., 2001; Dupont and Altenbach, 2003). The most effective environmental factor on wheat quality is N fertilization. At the same time, the degree of influence is affected by annual weather conditions and by residual soil N (López-Bellido et al., 2001). Therefore, proper management of N fertilizer is essential to ensure high quality wheat production. Design of fertilizer application regimes should combine rate, timing, splitting, and source of application, with a view to optimizing wheat yield and its quality (Borghi, 2000; Grant et al., 2001; Blankenau et al., 2002; Abedi et al., 2010). N fertilization increases the total quantity of flour proteins, resulting in an increase in both gliadins and glutenins (Luo et al., 2000; Panozzo and Eagles, 2000; Triboi et al., 2000; Johansson et al., 2001; Dupont and Altenbach, 2003; Martre et al., 2003; Johansson et al., 2004). However, the albumins-globulins content is scarcely influenced by N nutrition (Johansson et al., 2001; Dupont and Altenbach, 2003; Pedersen and Jorgensen, 2007; Fuertes-Mendizábal et al., 2010). As Nitrogen fertilizer rates and timing of application are a decisive factor in the obtaining of high yields, increased protein content and improved grain quality, numerous studies have been done in order to determine the optimum rate and time of N application (Borghi, 2000; Wooding et al., 2000; López-Bellido et al., 2001; Blankenau et al., 2002; Tea et al., 2004; Garrido-Lestache et al., 2005; Marino et al., 2009; Cui et al., 2010;). It is shown by some researches that applications of N later in the season (spring) and near anthesis is more effective in enhancing grain protein content in wheat than earlier applications (Ottman et al., 2000; Bly and Woodard, 2003). The goal of the present study was to examine the effect of rate and timing of N applications on yield and its yield components of one of the most important wheat cultivars, Shiraz cultivar, in Bajgah region environmental condition. Besides, gluten content and grain protein concentration under these treatments were assessed to suggest a suitable amount of N application and its proper timing to local farmers in order to obtain the highest yield with acceptable protein quality. So, the most significant aim of this study is to reduce the risk of over N application for environment in Iran farming system, what causes a deep concern among environmentalists, in aid of a practical result for farmers. In addition, total protein banding pattern in various vegetative growth stages and seed of wheat was examined to find whether polymorphism of seed protein and leaf protein especially the proteins which cooperate in photosynthesis reaction influenced by N treatments or not.

Materials and methods

Field experiments

From 2007 to 2008, the field experiment was conducted on silty loam soil at the Experimental Farm of School of Agriculture, Shiraz University, Shiraz, Iran, is located at bajgah (1810 m above the sea level with longitude 52º 35' and latitude 39º 40'). The experiment was laid out in a split-split plot design with four replications. The treatments included nitrogen at four levels (0, 120, 240 and 360 kg N ha $^{-1}$) and different nitrogen timing (table 1). After land preparation plowing, disking and ridging the plots (6 m long and 2 m wide) were done and winter wheat (CV. Shiraz) was planted at seeding rate of 180 kg ha $^{-1}$ in the rows. Spikes per plant were determined from 1 m^2 area of each plot by clipping the plants at the soil surface. After over drying and weighing, grain was threshed from the straw, cleaned, and weighed. Kernel weight was determined by counting weighing four 250 kernel samples taken from the harvested grain of each plot. Seeds per spike were calculated by dividing the number of kernels per harvested area by the number of spikes per harvested area. After harvesting the grain yield and yield components (spikes number m^{-2}, seeds number spike^{-1} and 1000 grain weight) were determined for all plots. Samples from all replications in different growth stages were used to determine the gluten and protein content and electrophoresis analyses.

Protein extraction

In different growth stages, the leaves were harvested, frozen in liquid nitrogen and then stored at -80° C. The frozen leaves were ground to a fine powder in liquid nitrogen and total protein extracted with ice-cold 0.1 M Tris - HCl buffer (pH 7.5) containing 5% (w/v) PVP (4:1 buffer volume/fresh weight). The homogenate was centrifuged at 13000 g for 15 min at 4° C, and the supernatant was used for determining protein concentration and SDS-PAGE analysis.
Then, the seeds were ground to a fine powder and 100 mg of powder was used for protein extraction. 1 ml extraction buffer was added to each micro tube containing 100 mg powder and centrifuged at 13000 g for 10 min at 4° C.

Protein measurement

The concentration of protein was measured by the Bradford method (1976). BSA was used as a standard.

Table 1. Nitrogen timing treatments.

	Sowing	Tillering	Stem elongation	Grain filling
T1	1/3	1/3	1/3	0
T2	0	1/3	1/3	1/3
T3	1/3	0	1/3	1/3
T4	1/3	1/3	0	1/3

SDS-PAGE

Prior to electrophoresis, samples were mixed with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% [w/v] glycerol, 5% [v/v] β-mercaptoethanol, 0.001% [w/v] bromophenol blue) and boiled for 5 min. Protein samples were separated on 5- 20% SDS- PAGE gradient gels based on method of Laemmli (1970). Staining and destaining of the gels were carried out by the standard Coomassie brilliant blue G-250 (CCB) staining method.

Gluten measurement

Flour was hand washed according to the standard method (AACC 1983) with 30 min of resting time. Isolated drying gluten contained was evaluated.

Statistical analysis

The experiment was laid out in a split- split plot design with four replications. All data were analyzed by analysis of variance (ANOVA) procedures using MSTATC software package. Treatment means were separated by Duncan's multiple range tests at (Duncan 0.05).

Results

Yield and yield components

Data in table 2 shows that, the different N rates (120, 240 and 360 kg ha^{-1}) have a significant effect on grain yield increasing (46% at N_{120}, 72% at N_{240}, and 78% at N_{360}) compared to control. However, there was no significant difference between 360 and 240 kg N ha $^{1-}$ treatments on grain yield. Results reveal a significant difference between grain yields in different nitrogen timing treatments. As data showed, T2 (nitrogen application in tillering, steam elongation and grain filling) as well as T4 (nitrogen application in sowing, tillering and grain filling) had the most effect on grain yield increasing (Table 2). Result of N rates and its timing interaction effects indicates that maximum wheat grain yield was obtained from 240 kg N ha $^{-1}$ when it was completely used before grain filling in three stages, T1, (8230 kg ha $^{-1}$) (Table 2).Results clearly indicate that N fertilizer had a significant effect on yield components (spikes number/ m^2, seeds number spike^{-1} and 1000 grain weight) and grain yield (Table 3). These parameters significantly enhanced with increasing nitrogen levels, but there was no significant increase from 240 to 360 kg N ha $^{-1}$ except seed number spike $^{-1}$ (Table 3). Table 4 shows that the highest spike number/m^2 was observed in T2 (no fertilizing in sowing time). However, T3 (no fertilizing in tillering stage) contribute to the minimum spike number/ m^2. In addition, no N application in tillering stage (T3 treatment) contributed to the fewest seed number/ spike. Data revealed that the minimum of 1000 grain weight was obtained in T1 (no N fertilization in grain filling period).

Table 2. Interaction effects of nitrogen rate and timing on wheat grain yield (kg ha $^{-1}$).

Treatment	Timing				
Nitrogen (kg ha $^{-1}$)	T1	T2	T3	T4	Mean
control	3930.00 h	3320.00 i	3850.00 hi	4700.00 g	3950.00 c
120	4400.00 bh	7130.00 bc	5560.00 ef	6030.00 de	5780.00 b
240	8230.00 a	7620.00 b	5000.00 fg	6400.00 de	6810.00 a
360	6530.00 cd	7430.00 b	6670.00cd	7430.00 b	7020.00 a
Mean	5775.00 b	6375.00 a	5270.00 c	6140.00 a	

Means in the same column and the last row followed by the same letters are not significantly different (P<0.05), according to Duncan's test.

Table 3. Effect of nitrogen rate on wheat yield and yield components.

Nitrogen (kg ha $^{-1}$)	Grain yield (kg ha $^{-1}$)	Spike number/ m^2	Spike seed number $^{-1}$	1000 grain Weight (g)
control	3950.00 c	550.58 c	21.47 d	37.50 c
120	5780.00 b	654.42 b	24.04 c	39.92 b
240	6810.00 a	675.08 ab	28.53 b	44.83 a
360	7020.00 a	690.67 a	33.29 a	45.83 a

Means in the same column followed by the same letters are not significantly different (P<0.05), according to Duncan's test.

Table 4. Effect of nitrogen timing on wheat yield and yield components.

Nitrogen timing	Grain yield (kg ha $^{-1}$)	Spike number/ m^2	Spike seed number $^{-1}$	1000 grain Weight (g)
T1	5775.00 b	642.92 b	27.42 a	38.25 b
T2	6375.00 a	690.00 a	27.33 a	44.08 a
T3	5270.00 c	581.67 c	27.17 b	42.50 a
T4	6140.00 a	656.17 b	28.42 a	43.25 a

Means in the same column followed by the same letters are not significantly different (P<0.05), according to Duncan's test.

Protein and gluten content

Analysis of the data revealed no significant effects of N rates and its timing on leaf protein content in different growth stages (data not shown). However, seed protein content was significantly affected by N rates (p< 0.01). Application of 240 kg N ha $^{-1}$ resulted in the maximum amount of seed protein content in all timing treatments (Fig. 1). The influence of N rates on the gluten was insignificant (Fig. 2). At the same time, nitrogen timing has a significant effect, too (p< 0.01). The maximum amounts of gluten were obtained from T2 (no fertilizing in sowing time), and T1 (no fertilizing in grain filling period) had significant negative effects on gluten (Fig. 2).

SDS-PAGE protein analysis

Figure 3 illustrates the SDS PAGE protein profile of wheat leaves in different growth stages and wheat seed under different N rate and its timing treatments. The results clearly showed that there is no polymorphism between different treatments in growth stages and seed. The intensity of band was measured densitometrically and it revealed that in growth stages, intensity of a 51 kDa band was increased in the most T_2 and T_3 treatments (Fig. 3).

Discussion

The relative low cost of N fertilizers and the misunderstanding of the relationship between N fertilizer rates and timing with crop yields have encouraged some farmers to over-fertilize winter wheat with N in Iran. Nitrogen fertilizer applications that exceed crop N requirements lead to environmental pollution including nitrate N leaching and N gaseous emissions. As a result, it is so essential to determine the plant response to N fertilization and its real N demand to develop rational practices for more N use efficiency in this plant, in order to improve the nitrogen application management (N rate and its timing) that may lead to achieve the highest yield with decline in environmental hazards. Results of present study show the increase in the N rate up to 240 kg ha $^{-1}$ had a positive effect on yield components that led to grain yield enhancement (Table 2). These results agree with those reported by Marino et al. (2009). It is noticeable that application above 240 kg N ha $^{-1}$ to 360 kg N ha $^{-1}$ did not influence yield component and grain yield significantly, which should be considered in N application management in order to decrease the environmental risk besides farming cost. It can be seen from Table 2 that the maximum amount of grain yield (8230 kg ha $^{-1}$) was obtained when 240 kg N ha $^{-1}$ was applied in vegetative growing stages (T1), while removing the fertilization in grain filling period did not affect it negatively. These results are in accordance with the report of Brown and Petrie (2006). Furthermore, investigation the effect of N timing on yield components revealed the lowest number of spike m^{-2} and seed per spikes were obtained in T3 (No N fertilization in tillering stage) which verifies this claim that in Triticum species, the number of spikes per unit area is set before stem elongation (Li et al., 2001) so N fertilization in tillering stage has a significant impact on this element. Besides, no N application in grain filling period has the most negative influence on 1000 grain weight (Table 4). Data shows that the highest values of all analyzed grain compone-

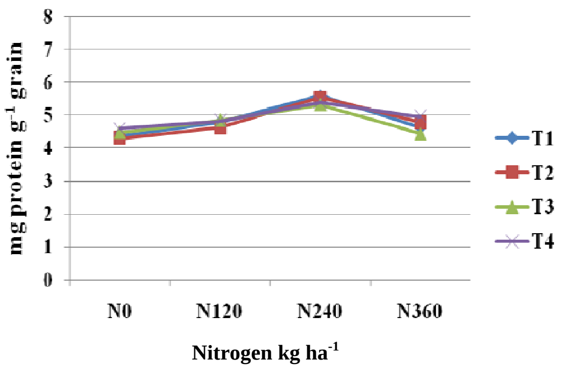

Fig 1. Effect of N fertilization rate and its timing on seed water soluble protein. T1: 1/3 N in sowing, 1/3 N in tillering, 1/3 N in stem elongation and 0 N in grain filling T2: 0 N in sowing, 1/3 N in tillering, 1/3 N in stem elongation and 1/3 N in grain filling T3: 1/3 N in sowing, 0 N in tillering, 1/3 N in stem elongation and 1/3 N in grain filling T4: 1/3 N in sowing, 1/3 N in tillering, 0 N in stem elongation and 1/3 N in grain filling.

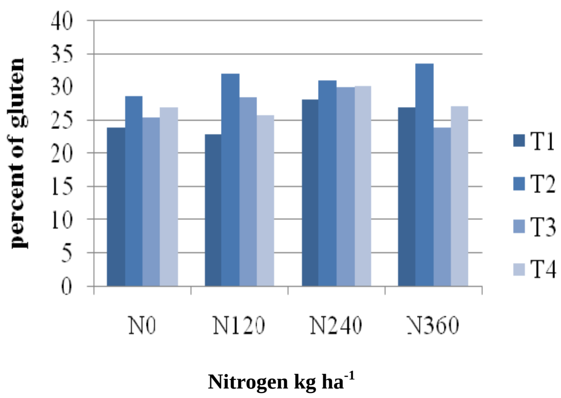

Fig 2. Effect of N fertilization rate and its timing on seed gluten content. T_1: 1/3 N in sowing, 1/3 N in tillering, 1/3 N in stem elongation and 0 N in grain filling T_2: 0 N in sowing, 1/3 N in tillering, 1/3 N in stem elongation and 1/3 N in grain filling T_3: 1/3 N in sowing, 0 N in tillering, 1/3 N in stem elongation and 1/3 N in grain filling T_4: 1/3 N in sowing, 1/3 N in tillering, 0 N in stem elongation and 1/3 N in grain filling.

nts were recorded at T2 (No N application at sowing time). Our present study clearly demonstrates that the amount of N fertilization before wheat planting was completely unnecessary, and was likely to move beyond the root zone, particularly under irrigated conditions. Similar results also were observed in other researches (López -Bellido et al., 2005; Chen et al., 2006; Cui et al., 2010). Overall, these results illustrated the yield components that are effective in seed number, are sensitive to N application in vegetative growth stages specially tillering. However, 1000 grain weight is significantly influenced by N fertilization in grain filling. Increasing yield potential without negative effect on grain quality is difficult, mainly because increases in grain yield are generally accompanied by a decrease in the grain's protein content, which is strongly associated with bread-making quality. Therefore, a proper management of N fertilizer is essential to ensure high quality wheat production. Design of fertilizer application regimes should combine rate, timing and splitting and source of application, with a view to optimizing wheat yield, its quality as well as environment conservation (Grant et al., 2001). It has been reported that N fertilization has the most effect on wheat quality (López -Bellido et al., 2001). However, the degree of influence is governed by annual weather conditions and by residual soil N. In order to investigate a proper N application management that contributes to a suitable wheat grain quality as well as its yield production, leaf and seed proteins were analyzed. Leaf protein amount showed no significant differences in different N rate and its timing treatments. It reveals that leaf protein is not influenced by N treatments. The same result was observed in other researches (Abedi et al., 2010; Rajala et al., 2009). At the same time, the maximum amount of seed total protein was observed in 240 kg N ha^{-1} in all timing treatments (Fig. 1). Similarly, other scientists reported that grain N content increased as applied N increased (Staggenborg et al., 2003; Garrido-Lestache et al., 2004; Garrido-Lestache et al. 2005; Bakht et al., 2009). It must be pointed out that over N application to 360 kg ha^{-1} decreased the seed protein content. Therefore, over N application should be avoided. Illustrations of SDS PAGE in Figure 3 show there was no polymorphism in leaf protein pattern in all growth stages, but the density of a 51 kDa band which may be related to Ribulose-1, 5-bisphosphate carboxylase activase (Rubisco activase) was increased in some treatments. This enzyme can alter the activity of Ribulose-1, 5-bisphosphate carboxylase/ oxygenase (Rubisco), a key enzyme that initiates both photosynthetic and photorespiratory carbon metabolism, by facilitating the dissociation of tightly bound sugar-phosphates from Rubisco in a process that requires ATP hydrolysis (Spreitzer and Savucci, 2002). It is now clearly established that grain protein quality is determined genetically for each wheat variety. In other words, the genetic effect is mainly reflected by qualitative variation such as protein polymorphism. In contrast, the environmental growth conditions (growing season, site, fertilization and so on) influence only on the quantity of these proteins (Wieser et al., 2008). Furthermore, seed water soluble proteins such as Albumin and Globulin showed no polymorphism (Fig. 3). Similar result was obtained in previous research (Abedi et al., 2010), which confirmed insensitivity of these proteins to nitrogenous fertilization. (Johansson et al., 2001; Dupont and Altenbach, 2003; Pedersen and Jorgensen, 2007; Fuertes-Mendizábal et al., 2010). With regard to this fact that the amount of protein is not the only factor affecting quality of wheat, and other factors such as protein composition can also play significant role (Johansson et al., 2001), at present experiment seed gluten as the most important factor in bread making quality was measured. Results in figure 2 illustrate that N rate did not have a significant effect on gluten content. As noted previously, protein composition of the wheat grain that play a key role in bakery quality is influenced by genotype, as well as by cultivation system and environmental conditions. In other words, although increased nitrogen supply correlated significantly to an increase in all protein components, its effect on grain protein quality also depends on the cultivar sown, due to different uses of available soil N. At the same time, N timing treatments influenced it significantly. These results agree with those reported by Garrido-Lestache et al., 2005. Omitting N application in sowing time (T2) had the least negative effect on gluten content. In other word, the maximum amount of gluten was recorded at T2. It confirms N application in sowing time has an effective role on seed germination and plant settlement while seed quality is not influenced significantly. As seen in figure 2, gluten content showed its lowest amount when N

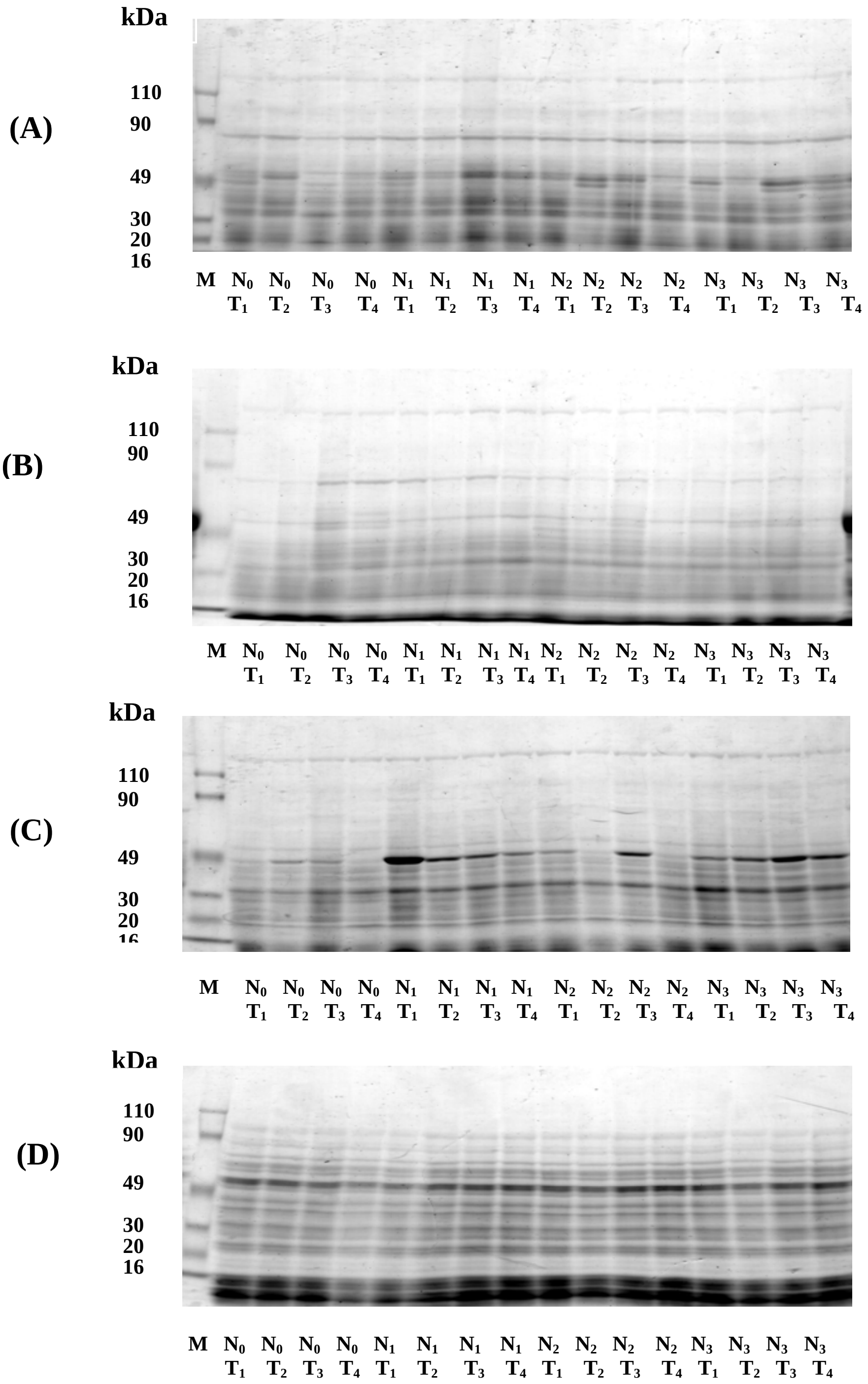

Fig 3. Water soluble protein banding patterns of wheat in (A) tillering stage, (B) stem elongation stage, (C) spike emergence stage and (D) seed; M: Protein marker; N0: 0 kg N ha^{-1} N1: 120 kg N ha^{-1} N2: 240 kg N ha^{-1} N3: 360 kg N ha^{-1}
T1: 1/3 N in sowing, 1/3 N in tillering, 1/3 N in stem elongation and 0 N in grain filling
T2: 0 N in sowing, 1/3 N in tillering, 1/3 N in stem elongation and 1/3 N in grain filling
T3: 1/3 N in sowing, 0 N in tillering, 1/3 N in stem elongation and 1/3 N in grain filling
T4: 1/3 N in sowing, 1/3 N in tillering, 0 N in stem elongation and 1/3 N in grain filling

fertilization was removed in grain filling period (T1). Because gluten components, gliadin and glutenin, are defined as proteins that accumulate during the grain-filling period (Shewry and Halford, 2002). Previous studies have shown that late application of N increased protein content in bread wheat (Luo et al., 2000; Ottman et al., 2000; Weber et al., 2008). Therefore, it is exactly clear that late season N application can improve seed quality efficiently. At the same time, conditions that shorten grain-filling, such as high temperature or drought, can affect the balance of protein fractions as well (Jamieson et al., 2001; Garrido-Lestache et al., 2005). In conclusion, nitrogen fertilization management (rate and timing) offers the opportunity for increasing wheat protein content and its quality besides high wheat production. The present study indicates it is possible to obtain maximum grain yield and protein just in 240 kg ha^{-1} nitrogen level. However, excess applications of N are not economically efficient and can reduce protein content as well as create environmental problems. In addition, not only increasing the N fertilization rate but also N timing had a beneficial effect on grain yield and its quality. The N applied during vegetative growth stages led to increase in yield and late season N resulted in gluten enhancement.

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