Australian Journal of Crop Science AJCS

AJCS 6(12):1624-1629 (2012) ISSN:1835-2707

Effects of nitrogen fertilisation rate on the accumulation of high-molecular-weight glutenin subunits and distribution of glutenin macropolymer size in strong gluten wheat (*Triticum aestivum* L.)

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Abstract

The high-molecular-weight glutenin subunits (HMW-GS) and glutenin macropolymer (GMP) in wheat grain significantly affect the wheat quality. The role of nitrogen in determining grain yield and quality has been extensively studied. However, the effects of nitrogen application on HMW-GS accumulation and GMP particle distribution are rarely reported. In the present study, the strong gluten wheat cultivar Shannong 12 was used in a field experiment to examine the effects of the nitrogen fertilisation rate on HMW-GS accumulation and GMP size distribution. The total and individual HMW-GS contents both increased with nitrogen application 14 days after anthesis. The responses of individual HMW-GS contents to nitrogen application varied; subunits 5 and 10 were more sensitive to nitrogen application than subunit 15. The diameter of the GMP particles in wheat grain ranged from 0.37 μm to 245 μm. The distribution of number percentage exhibited a one-peak curve at 1 μm, which indicated that most of the GMP particles in the wheat grains had small diameters. The distributions of volume percentage and surface area percentage of GMP exhibited two-peak curves. The number and volume arcsine square roots of GMP particles larger than 100 μm increased with nitrogen application, whereas those of GMP particles smaller than 10 μm decreased. The HMW-GS and GMP contents were significantly positively correlated with GMP particles larger than 100 μm. These results suggested that nitrogen application promoted the development of GMP and increased the percentage of larger GMP particles, thereby enhancing wheat quality.

Keywords: Glutenin macropolymer; High-molecular-weight glutenin subunits; Nitrogen fertilisation rate; Wheat (*Triticum aestivum* L.).
Abbreviations: DAA, days after anthesis; GMP, glutenin macropolymer; HMW-GS, high-molecular-weight glutenin subunits; N0, 0 kg hm^{-2} nitrogen application; N120, 120 kg hm^{-2} nitrogen application rate; N240, 240 kg hm^{-2} nitrogen application rate; N360, 360 kg hm^{-2} nitrogen application rate; SDS, sodium dodecyl sulphate.

Introduction

Glutenin is an important component of wheat (*Triticum aestivum* L.) protein that determines dough flexibility and is closely associated with bread-baking properties (Sapirstein and Fu, 1998; Tronsmo et al., 2002). Glutenin polymer consists of a series of polymers with different molecular weights, including the sodium dodecyl sulphate (SDS)-insoluble glutenin fraction known as glutenin macropolymer (GMP). Studies show that the GMP amount has a greater effect on the flour bread-making quality than soluble glutenin polymer (Steffolani et al., 2008). GMP is found in wheat endosperm as spherical particles that range in diameter from 1 μm to 300 μm (Don et al., 2003a). The content and size distribution of GMP particles are affected by genetic and environmental factors. GMP can be further divided into high-molecular-weight (HMW-GS) and low-molecular-weight (LMW-GS) glutenin subunits. The composition and expression of HMW-GS and LMW-GS determine the GMP content (Gianibelli et al., 2001). HMW-GS plays a key role in the formation of GMP particles, and is closely related to the size and quantity of insoluble glutenins. Weegels et al. (1997) found that the insoluble glutenin content in wheat grain increases with increased HMW-GS. Jiang et al. (2009) observed that the variation in GMP content with various water-stress treatments is attributed to changes in HMW-GS accumulation in the grain. In addition to genetic factors, wheat quality is also affected by cultivation conditions such as nitrogen application (Triboi et al., 2000). However, the effects of nitrogen application on HMW-GS accumulation and GMP particle distribution are rarely reported. In the current study, a winter wheat cultivar, Shannong 12, was grown under different nitrogen treatments. The aim was to determine the relationship between HMW-GS accumulation and GMP particle properties. The effects of the nitrogen fertilisation rate on the HMW-GS content and on glutenin formation were also analysed.

Results

Effect of the nitrogen fertilisation rate on the HMW-GS content

HMW-GS accumulation started approximately 14 days after anthesis (DAA) and then increased 14 DAA; significant differences were observed among different nitrogen application rates (Fig. 1). The HMW-GS content gradually increased from 14 DAA to 28 DAA, and then rapidly increased thereafter

Table 1. Effects of the nitrogen fertilisation rate on the number, volume, and surface area distribution of GMP particles in wheat grains during the 2009–2010 and 2010–2011 growing seasons ($sin^{-1} P^{1/2}$).

Season	Nitrogen fertilisation rate	GMP particle diameter (μm)								
		Number			Volume			Surface area		
		<10	10–100	>100	<10	10–100	>100	<10	10–100	>100
2009–2010	N0	88.22a	1.78a	0	43.85a	45.45d	6.33d	72.54a	17.43c	0.51c
	N120	88.05a	1.89a	0.57a	28.02b	51.88b	23.58c	64.13b	24.87b	6.64b
	N240	87.59a	2.37a	0.57a	24.37d	53.46a	25.39b	60.67c	27.97a	8.12a
	N360	87.78a	2.17a	0.57a	25.95c	49.97c	28.10a	62.82b	25.72b	8.20a
2010–2011	N0	88.15a	1.85b	0	44.15a	45.64d	3.49d	72.10a	17.88c	0.79c
	N120	87.57a	2.36a	0.57a	30.75b	52.96b	18.55c	64.52b	24.84b	5.31b
	N240	87.50a	2.43a	0.57a	25.22d	55.72a	21.62b	60.87d	27.90a	7.68a
	N360	87.50a	2.43a	0.57a	26.70c	50.84c	26.34a	63.12c	25.52b	7.87a

Notes: N0, N120, N240, and N360 represent the nitrogen application rates of 0, 120, 240, and 360 kg hm^{-2}, respectively; <10, 10–100, and >100 represent the GMP particle diameters <10 μm, 10 μm to 100 μm, and >100 μm, respectively; data with the same letters are not significantly different (P<0.05).

Table 2. Correlation coefficients among the GMP volume distribution, total HMW-GS content, and GMP content.

Item	GMP particle diameter (μm)		
	<10	10–100	>100
Total HMW-GS content (μg mg^{-1})	–0.876**	0.569	0.928**
GMP content (mg g^{-1})	–0.919**	0.659	0.944**

Notes: <10, 10–100, and >100 represent the GMP particle diameters <10 μm, 10 μm to 100 μm, and >100 μm, respectively; **significant level of 0.01.

(Fig. 2). The HMW-GS contents of the 0, 120, 240, and 360 kg hm^{-2} nitrogen applications (N0, N120, N240, and N360, respectively) after 21 DAA were significantly different. The HMW-GS contents were ranked in the following order: N360 > N240 > N120 > N0. In contrast to N0, the HMW-GS contents at N120, N240, and N360 in 2009–2010 increased by 13.02%, 25.02%, and 25.77%, respectively. Similarly, the HMW-GS contents at N120, N240, and N360 in 2010–2011 increased by 12.95%, 30.01%, and 38.42%, respectively. The individual HMW-GS contents in wheat grains exhibited a similar pattern with the total HMW-GS content (Fig. 3). The individual HMW-GS contents increased during wheat grain development in the order of subunit 5 > 10 > 14 > 15. The content of each subunit in treatments N240 and N360 was significantly different from that in N0 after 21 DAA. Different response patterns of the individual HMW-GS contents to the nitrogen fertilisation rate were observed among the nitrogen treatments. Nitrogen application increased the average individual HMW-GS content by 33.84% (subunit 5), 31.92% (subunit 10), 25.91% (subunit 14), and 6.58% (subunit 15) in 2009–2010, as well as by 32.94% (subunit 5), 31.75% (subunit 10), 26.26% (subunit 14), and 17.12% (subunit 15) in 2010–2011. These results indicated the variations in the individual HMW-GS contents in response to nitrogen application.

Effect of the nitrogen fertilisation rate on the GMP content

The dynamic changes in GMP content after anthesis are shown in Fig. 4. The GMP content initially increased prior to 14 DAA, decreased before reaching 28 DAA, and finally increased 35 DAA. This trend may be attributed to the lower protein accumulation rate than the starch accumulation rate, which had a dilutive effect on the GMP content. The GMP content in treatments N120, N240, and N360 significantly differed from that in N0 after 7 DAA. Nitrogen application increased the GMP content by 17.72% (N120), 30.45% (N240), and 34.54% (N360) in 2009–2010, as well as by 14.16% (N120), 27.40% (N240), and 36.99% (N360) in 2010–2011 relative to the non-nitrogen treatment at maturity. These results showed that an appropriate application of nitrogen fertiliser can significantly increase the GMP content.

Effect of the nitrogen fertilisation rate on the GMP particle distribution

GMP particle number distribution

The number distribution of GMP particles exhibited a single-peak curve with the maximum at 1 μm, which indicated that most GMP particles in the wheat grains had small diameters (Fig. 5A). The GMP particles mainly consisted of small particles (<10 μm) that accounted for 87.59 to 88.22 (2009–2010) and 87.50 to 88.15 (2010–2011) of the total particles (Table 1). The medium (10 μm to 100 μm) and large (>100 μm) GMP particles only accounted for 1.78 to 2.37 (2009–2010), 1.85 to 2.43 (2010–2011), and 0 to 0.57 (2009–2010, 2010–2011) of the total particles (Table 1). Nitrogen application significantly increased the number arcsine square root of the 10 μm to 100 μm and >100 μm GMP particles compared with N0. However, no significant difference was observed among the different nitrogen treatments.

GMP particle volume distribution

The volume distribution of the GMP particles exhibited a two-peak curve, with particle diameters of 5 and 60 μm to 100 μm at each peak (Fig. 5B). The volume arcsine square root of the <10 μm GMP particles ranged from 24.37 to 43.85 (2009–2010) and 25.22 to 44.15 (2010–2011) of the total particles; those of the 10 μm to 100 μm were 45.45 to 53.46 (2009–2010) and 45.64 to 55.72 (2010–2011) of the total particles; and those of the >100 μm GMP particles were 6.33 to 28.10 (2009–2010) and 3.49 to 26.34 (2010–2011) of the total particles (Table 1). The volume arcsine square root of the <10 μm GMP particles decreased, whereas that of the 10 μm to 100 μm GMP particles increased with increased nitrogen fertilisation rate from N0 to N240. The volume arcsine square root of the >100 μm particles significantly increased with increased nitrogen fertilisation rate from N0 to N360. These findings indicated that the amount of large GMP particles increased and that of smaller GMP particles decreased with

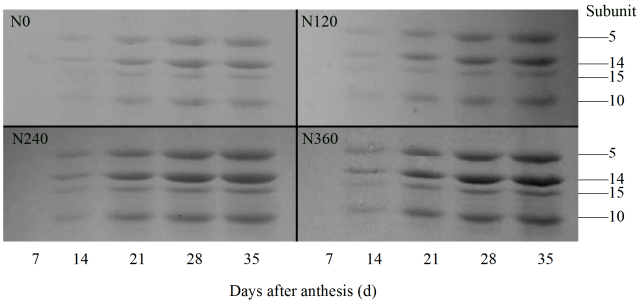

Fig 1. SDS-PAGE of HMW-GS to determine the accumulation dynamics in wheat grains under different nitrogen fertilisation rates after anthesis during the 2010–2011 growing season. N0, N120, N240, and N360 represent the nitrogen application rates of 0, 120, 240, and 360 kg hm^{-2}, respectively. The numbers 5, 14, 15, and 10 represent the individual subunits of 5 subunits, 14 subunits, 15 subunit, and 10 subunits, respectively. 14 DAA: the onset of increasing HMW-GS content for wheat; 28 DAA: the onset of quickly increasing HMW-GS content for wheat.

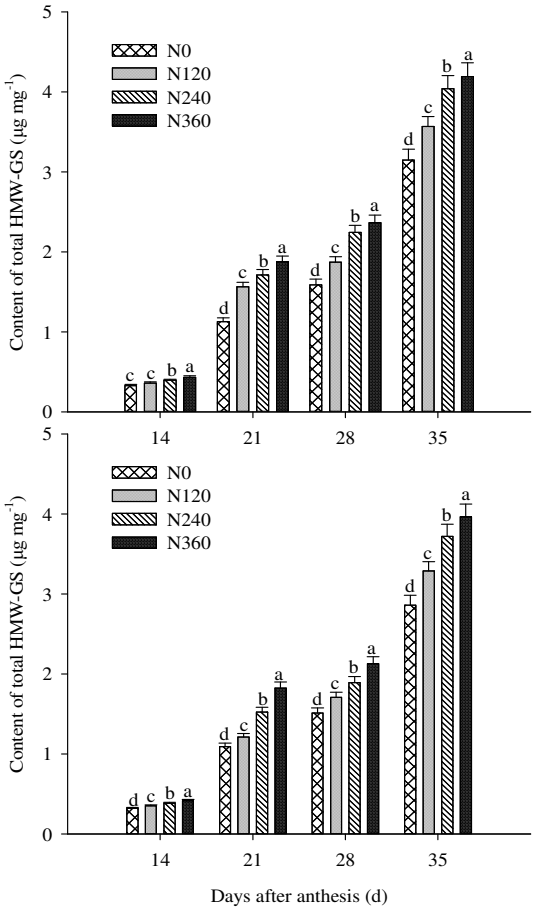

Fig 2. Effects of the nitrogen fertilisation rate on the HMW-GS content in wheat grains after anthesis in the 2009–2010 (top) and 2010–2011(bottom) growing seasons. N0, N120, N240, and N360 represent the nitrogen application rates of 0, 120, 240, and 360 kg hm^{-2}, respectively. 14 DAA: the onset of increasing HMW-GS content for wheat; 28 DAA: the onset of quickly increasing HMW-GS content for wheat. Bars (with standard errors) with the same letters are not significantly different (P<0.05).

nitrogen application. The development of GMP particles may also be promoted by nitrogen application.

GMP particle surface area distribution

The surface area distribution of GMP particles exhibited a two-peak curve, with the peaks at 2 and 40 μm to 80 μm (Fig. 5C). The surface area arcsine square roots were 60.67 to 72.54 (2009–2010) and 60.87 to 72.10 (2010–2011) of the total particles for the small GMP particles (<10 μm); 17.43 to 27.97 (2009–2010) and 17.88 to 27.90 (2010–2011) of the total particles for the medium particles (10 μm to 100 μm); and 0.51 to 8.2 (2009–2010) and 0.79 to 7.87 (2010–2011) of the total particles for the large particles (>100 μm) (Table 1). The surface area arcsine square root of the small GMP particles decreased and that of the medium particles increased, with increased nitrogen fertilisation rates from N0 to N240. The surface area arcsine square root of the large GMP particles significantly increased with increased nitrogen fertilisation rate from N0 to N360.

Correlation analysis

The HMW-GS and GMP contents were significantly negatively correlated with the volume arcsine square root of the <10 μm GMP particles, but were significantly positively correlated with that of the >100 μm particles. These results indicated that larger GMP particles have higher HMW-GS contents (Table 2).

Discussion

Effect of nitrogen on HMW-GS accumulation

The HMW-GS content is significantly affected by the cultivar, water, temperature, soil condition, and fertiliser (Deng et al., 2008; Irmak et al., 2008; Yue et al., 2007). In this study, the HMW-GS content increased during wheat grain development. Compared with the non-nitrogen treatment, nitrogen application increased the HMW-GS content. This result indicated that nitrogen promoted HMW-GS accumulation. The change trend of the individual HMW-GS contents was similar to that of the total HMW-GS content. Subunits 5 and 10 significantly increased with nitrogen application. This finding indicated the considerable difference in the responses of individual HMW-GS to nitrogen application; subunits 5 and 10 are more sensitive to nitrogen application than subunit 15.

GMP particle distribution characteristics and their relationship to HMW-GS accumulation

Don et al. (2005a, 2006) showed that GMP existed in wheat endosperm as spherical particles. In the present study, the GMP particle diameters ranged from 0.37 μm to 245 μm. The number distribution showed that GMP mainly consisted of <10 μm particles. The volume distribution showed that GMP mainly consisted of 10 μm to 100 μm particles. The surface area distribution showed that GMP mainly consisted of <10 μm particles. HMW-GS is closely related to glutenin polymers in terms of molecular weight. Flour from the 5+10 variety is shown to have larger glutenin particles than flour from the 2+12 variety (Wang et al., 2004; Gupta et al., 1993). In the present study, the HMW-GS content was negatively correlated with the volume arcsine square root of small GMP particles but positively correlated with large GMP particles. This result indicated that larger GMP particles had higher HMW-GS contents, which may have resulted from the higher number of

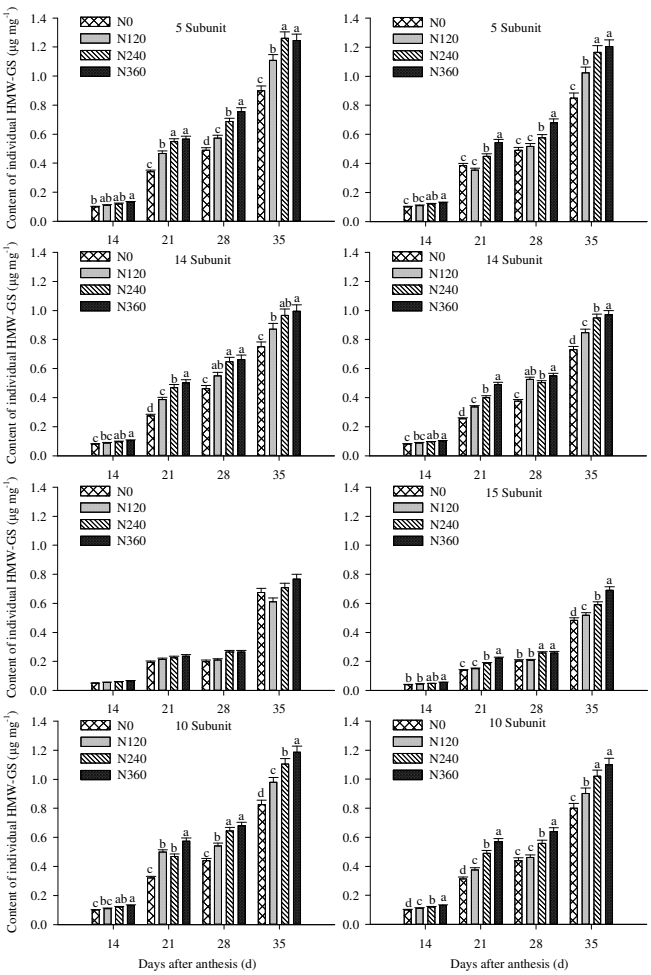

Fig 3. Effects of the nitrogen fertilisation rate on the individual HMW-GS contents in wheat grains after anthesis during the 2009–2010 (left) and 2010–2011 (right) growing seasons. N0, N120, N240, and N360 represent the nitrogen application rates of 0, 120, 240, and 360 kg hm^{-2}, respectively. 14 DAA: the onset of increasing individual HMW-GS content for wheat; 28 DAA: the onset of quickly increasing individual HMW-GS content for wheat. Bars (with standard errors) with the same letters are not significantly different (P<0.05).

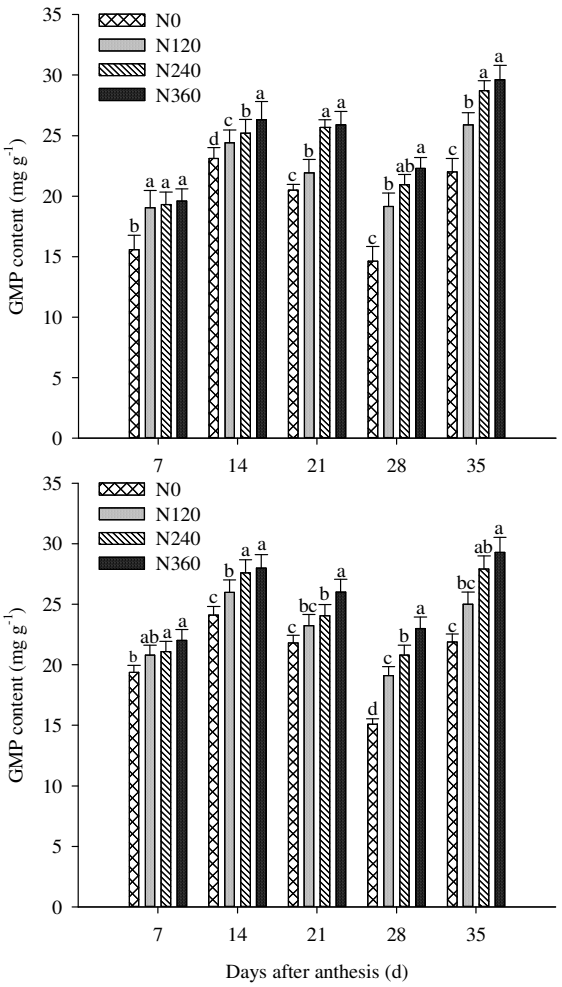

Fig 4. Effects of the nitrogen fertilisation rate on the GMP content in wheat grains after anthesis during the 2009–2010 (top) and 2010–2011 (bottom) growing seasons. N0, N120, N240, and N360 represent the nitrogen application rates of 0, 120, 240, and 360 kg hm^{-2}, respectively. 14 DAA and 35 DAA: the peaks of GMP content in wheat; 28 DAA: the trough of GMP content in wheat. Bars (with standard errors) with the same letters are not significantly different (P<0.05).

disulphide bonds in HMW-GS (Wieser, 2007). The formation of a glutenin particle involves three distinct steps (Don, 2005b): the biosynthesis of glutenin subunits, the formation of larger oligomer clusters, and further assembly into larger insoluble glutenin particles. The fusion of protein particles is also sometimes observable during maturation. High temperatures can slow down the biosynthesis of glutenin (first step), which can result in low GMP content. However, steps 2 and 3 are accelerated at high temperatures, which lead to the formation of larger particles. In the current study, nitrogen application promoted the biosynthesis of glutenin subunits (step one) and increased the HMW-GS content. The higher amount of HMW-GS accelerated step two, and oligomer clusters formed larger insoluble particles. However, no significant difference was observed in the number arcsine square root of GMP particles smaller than 10 μm upon nitrogen application. Whether nitrogen application promotes the fusion of small GMP particles requires further study. The formation of GMP particles was promoted by nitrogen application, which is an important factor that affects wheat quality enhancement.

Materials and methods

Experimental design

Field experiments were conducted at the experimental station of Henan Agricultural University, Zhenzhou, Henan province, PR China, in two growing seasons: from October 2009 to June 2010 and from October 2010 to June 2011. The strong gluten winter wheat cultivar Shannong 12 was used. A soil layer 0 cm to 20 cm thick contained 1.22% organic matter, 0.085% total nitrogen, 71.50 mg kg^{-1} available nitrogen, 44.23 mg kg^{-1} available phosphate, and 83.30 mg kg^{-1} available potassium. Four nitrogen treatments, namely, nitrogen fertilisation rates of

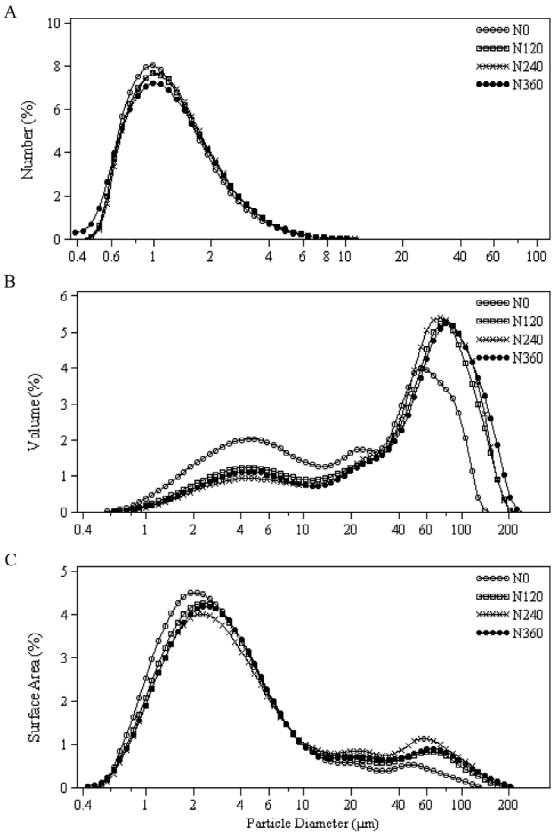

Fig 5. (A) Number, (B) volume, and (C) surface area distributions of GMP particles in wheat grains after anthesis during the 2010–2011 growing season. N0, N120, N240, and N360 represent the nitrogen application rates of 0, 120, 240, and 360 kg hm^{-2}, respectively. The number, volume, and surface area represent the distribution percentages of the number, volume, and surface area of the GMP particles, respectively. 1 μm: the peaks of number for GMP particles in wheat (A); 5 μm and 60 μm to 100 μm: two peaks of volume for GMP particles in wheat (B); 2 μm and 40 μm to 80 μm: two peaks of surface area for GMP particles in wheat (C).

0, 120, 240, and 360 kg hm^{-2}, were used. One-half of the total nitrogen was applied prior to sowing, whereas the other half was top-dressed at the wheat jointing stage. Basal fertilisers containing 125 kg hm^{-2} P_2O_5 and 112.5 kg hm^{-2} K_2O were used. Seeds were sown in a randomised block design on 14 October 2009 and 15 October 2010 in a 2.5 m × 2.5 m plot at a density of 150 plants m^{-2}. Each treatment had three replicates. Spikes that flowered on the same day were labelled. Forty labelled spikes were sampled 7, 14, 21, 28, and 35 DAA.

HMW-GS content measurement

HMW-GS was separated by SDS-PAGE according to the method described by Liang et al. (2002). The quantification method of HMW-GS was based on that of Yue et al. (2007). In a typical experiment, 20 mg of grain sample was defatted with isopropanol, incubated at 60 °C for 30 min with continuous shaking, and then centrifuged at 10 000*g* for 10 min. The sedimentation was reserved and then defatted with isopropanol. The entire procedure was repeated three times. The sedimentation was then mixed with 1 ml of extraction buffer, which contained 62.5 mmol l^{-1} Tris-HCl (pH 6.8), 100 mg g^{-1} glycerol, 20 mg g^{-1} SDS, and 50 mg g^{-1} β-mercaptoethanol. The mixture was incubated at 60 °C for 1 h with continuous shaking and then centrifuged at 10 000*g* for 10 min. The supernatant was then subjected to SDS-PAGE. The acrylamide concentrations in the resolving and stacking gels were 10 and 4 mg g^{-1}, respectively. Gluten in the extract (15 μl) was loaded into each lane. After electrophoresis, the gels were stained with 0.5 mg Coomassie Brilliant Blue R250 for 24 h, and then destained in distilled water for 48 h. Each band was then cut from the gel, extracted with 50 ml l^{-1} isopropyl alcohol containing 30 mg g^{-1} SDS, and then monitored at 595 nm using a UV-2401 spectrophotometer. During the preliminary electrophoresis, the wheat cultivars Chinese Spring (null, 7+8, and 2+12), Marquis (1, 7+9, and 5+10), and Xiaoyan6 (1, 14+15, and 2+12) were used as standards to identify the HMW-GS types in Shannong 12 (null, 14+15, and 5+10).

GMP content measurement

The GMP content was measured using the method described by Weegels et al. (1996) and Sun et al. (2001). A grain sample (0.05 g) was suspended in 1 mL of 1.5 mg g^{-1} SDS solution and then centrifuged at 15 500*g* for 30 min at 20 °C. The supernatant was decanted and the sediment was collected as GMP. The nitrogen content of the sediment was measured with the biuret reagent and taken as the GMP content.

GMP isolation and particles size analysis

GMP was isolated using 1.4 g of dispersing flour in 28 mL of 1.5 mg g^{-1} SDS solution and by centrifuging at 80 000*g* for 30 min at 20 °C (Don et al., 2003b). The supernatant was decanted and the gel layer was collected as GMP. The GMP gel (1 g) was transferred to a tube containing 10 ml of 1.5 mg g^{-1} SDS solution. The tube was kept at 4 °C for 3 h, during which the tube was vortexed every 15 min. The suspension (1 ml) was then transferred into the dispersion tank of a laser diffraction particle size analyser that contained double-distilled water for size measurements. The equivalent volume, equivalent surface area, and number proportions of the GMP granules were automatically obtained by the laser diffraction particle size analyser; the projected area represented the same volume, surface area, and number proportions as those of real GMP granules. The GMP particle size distributions of the diluted GMP dispersions were determined by laser diffraction using a Coulter LS13320 (Beckman Coulter Instruments, San Francisco, CA, USA).

Statistical analysis

All data were subjected to one-way ANOVA using the SPSS software. Correlation analyses between the GMP volume distribution and HMW-GS content were conducted using data sets.

Conclusions

Nitrogen application increased the HMW-GS and individual HMW-GS contents. Significant differences were found among the responses of individual HMW-GS contents to nitrogen application. The HMW-GS and GMP contents were significantly positively correlated with GMP particles larger than 100 μm. Nitrogen application promoted GMP particle formation and increased the number of large-volume particles,

which contributed to the improvement of wheat quality.

Acknowledgements

The authors wish to express their sincere thanks to Xinming Ma, Henan Agricultural University for their kind assistance in field experiments. This research was supported by grants from the Special Research Foundation of Agriculture Industry(201102001), and the System of Industrial Technologies for Modern Agriculture of Henan Province(S2010-01-G04)

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