

The Institute for Plant Nutrition and Soil Science  
of the Christian-Albrechts-Universität Kiel

**Composition, distribution and gene expression of  
storage proteins along with baking quality in winter  
wheat as affected by late-season nitrogen supply**

**Dissertation**

submitted for the Doctoral Degree

awarded by the Faculty of Agricultural and Nutritional Sciences

of the Christian-Albrechts-Universität Kiel

submitted by

M.Sc. Anne Roßmann

born in Kühlungsborn

Kiel, 2018

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I can do all this through Him who gives me strength.

The Holy Bible, Philippians 4:13

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### 1. General Introduction

#### 1.1. Nitrogen uptake, assimilation, transport and storage

Nitrogen (N) is an essential nutrient for plants, being involved in the synthesis of chlorophyll and being a basic constituent of proteins, nucleic acids, co-enzymes, phytohormones and secondary metabolites. After Carbon, oxygen and hydrogen, N is the element required by plants in largest amounts (Hawkesford et al., 2012). Therefore, N is crucial for plant growth and biomass production.

N is primarily absorbed by plants via the roots in form of ammonium or nitrate. N uptake via the leaves in form of molecular nitrogen ( $N_2$ ) or urea is also possible (Heldt and Piechulla, 2015). For the uptake of nitrate, plants use both high and low affinity transporters, which are encoded by two gene families: NRT1 and NRT2. The NRT1 transporters are responsible for the broad nitrate uptake and are expressed inducibly as well as constitutively while the NRT2 transporters contribute especially to the nitrate-induced high affinity nitrate uptake (Crawford and Glass, 1998).

When nitrate is absorbed from the soil solution by the roots, only a proportion of the nitrate is assimilated in the roots. The remaining part is transported through the xylem to be assimilated in the shoots (Forde, 2000; Tsay et al., 2007). The first step of nitrate assimilation is the reduction of nitrate to nitrite. This reaction is catalysed by the enzyme nitrate reductase and takes place in the cytosol. The generated nitrite is then transported to chloroplasts or root plastids, where it is further reduced to ammonium by the nitrite reductase. To avoid ammonium toxicity, plants transform the produced ammonium immediately into amino acids. For this conversion two enzymes are crucial: glutamine synthetase (GS) and glutamate synthetase (also known as glutamine-2-oxoglutarate

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aminotransferase (GOGAT)). GS combines ammonium with glutamate to produce glutamine. GOGAT, which is stimulated by high levels of glutamine, forms two molecules of glutamate by transferring the amide group of glutamine to 2-oxoglutarate. Ammonium can also be assimilated by the enzyme glutamate dehydrogenase (GDH), which forms glutamate using ammonium and 2-oxoglutarate (Taiz and Zeiger, 2006).

Nitrogen can be transported from the roots to the shoot in forms of nitrate, amino acids and amides via the xylem. In the phloem, N is translocated from the leaves to seeds (fruits), vegetative storage organs and roots in the form of amino acids and amides (Schopfer and Brennicke, 2010). When nitrate supply is high, the surplus nitrate can be stored in vacuoles. Amino acids, which are the products of nitrate assimilation, can be stored in form of proteins. Those storage proteins do not have enzymatic functions in plant cells and are accumulated in protein bodies (Heldt and Piechulla, 2015). The synthesis of grain storage proteins takes place on the membranes of the rough endoplasmic reticulum (ER). Then they are transported into the lumen and pass the Golgi apparatus into specific protein storage vacuoles (Shewry and Halford, 2002).

### **1.2. Nitrogen fertilisation practice in wheat**

To meet the needs of developing wheat plants, nitrogen fertiliser is applied in three or four doses during the growing season. The first application is usually carried out at the beginning of vegetation or at tillering (growth stage (GS) 25). The supply of N at this stage of plant growth improves the development of ears at side tillers and spikelets. The next time point when N is supplied is the end of tillering or start of stem elongation (GS 29/30) and is supposed to improve fertility. The third dose of N is applied at GS 39/49 (flag leaf visible/end of booting) and can be followed by a fourth application at anthesis (GS 61). While the third N



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application improves the process of grain filling, the fourth N application enhances grain quality (*Effizient düngen - Winterweizen*, 2018).

The major proportion of the required N is applied to the soil in forms of nitrate, ammonium or urea. However, foliar N application is advantageous especially for the later time points in the growing season. When supplying N via the leaves in form of urea farmers can reduce N losses due to denitrification and leaching compared to soil N applications. Furthermore N can be provided even though plant root activity might be reduced because of dry weather conditions (Gooding and Davies, 1992).

### **1.3. Wheat (*triticum aestivum* L.) – importance and history**

Wheat has first been cultivated between 9000 and 7000 BC in the forms of diploid einkorn and tetraploid emmer (Bell, 1987; Shewry, 2009). These two wheats developed from the domestication of natural populations but bread wheat, as we know it, arose by the hybridisation of cultivated emmer with the wild grass *Triticum tauschii*. At present, the vast majority of wheat grown worldwide is hexaploid bread wheat (95 %), the largest part of the remaining 5 % is tetraploid durum wheat (Shewry, 2009).

The worldwide wheat production increased massively over the past 50 years, with 222 million tonnes (t) being produced in 1961 and a production of 750 million tonnes in 2016. Unlike the extent of wheat production, the area used for wheat cultivation remained stable. In 1961, wheat was grown on 204 million hectares (ha), until 2016 this area has extended slightly to 220 million ha. To increase the wheat production on the same area was only possible because of the rise in the mean yield. While the world mean wheat yield was 1.1 tonnes per hectare ( $t\ ha^{-1}$ ) in 1961, in 2016  $3.4\ t\ ha^{-1}$  were harvested. In Germany, the yield improved by  $4.7\ t\ ha^{-1}$  during that period of time ( $2.9\ t\ ha^{-1}$  in 1961 and  $7.6\ t\ ha^{-1}$  in 2016). Nowadays, after maize, wheat is the biggest staple crop worldwide. A large proportion of

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wheat is used for food production. 18.3 % of the daily required energy supply and 19.5 % of the necessary protein intake is covered by wheat products (<http://www.fao.org/faostat/en/#compare>), demonstrates the importance of wheat and its quality for human nutrition.

### **1.4. Wheat storage proteins and their relation to baking quality**

Wheat storage proteins have been first isolated in 1745 by J. Beccari. A classification of plant proteins based on their solubility was established by T. B. Osborne in 1924 (Shewry and Halford, 2002). He categorised the wheat grain proteins into four groups: albumins (water soluble), globulins (soluble in dilute aqueous salts), gliadins (soluble in aqueous alcohols) and glutenins (soluble in dilute acids or alkalis and detergents) (Osborne, 1924). A new molecular classification of wheat prolamins (comprising gliadins and glutenins) considers their structural relationships: sulphur (S)-rich prolamins (monomeric  $\alpha$ - and  $\gamma$ -gliadins and polymeric low molecular weight glutenin subunits (LMW-GS), S-poor prolamins (monomeric  $\omega$ -gliadins) and high molecular weight (HMW) prolamins (polymeric high molecular weight glutenin subunits (HMW-GS)). The S-rich prolamins are the major components measured by quantity with proportions of about 70-80 % of the total prolamins. They contain high levels of cysteine and methionine as well as glutamine and proline. The S-poor prolamins make up about 10 % of the wheat prolamins and contain little or no cysteine and methionine but the levels of glutamine, proline and phenylalanine are usually high in this fraction. The HMW prolamins are relatively minor components with proportions up to about 10 % of total wheat prolamins. This fraction is characterised by high contents of glycine along with high proline and glutamine. Their polymers are stabilised by inter-chain disulphide bonds (Shewry and Casey, 1999).

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The proteins of wheat grain principally determine the bread-making quality of its flour. Both, the quantity and the composition of proteins influence the baking performance. Especially the gluten proteins are determining baking quality (Goesaert et al., 2005). As gliadins and glutenins contribute to different dough rheological properties a balanced ratio of these protein fractions is required for good baking quality. Gliadins are responsible for viscosity and the glutenins make the dough elastic (Zhao et al., 1999; Zörb et al., 2009). By forming a continuous network the glutenin polymers provide strength and elasticity to the dough (Belton, 1999). The monomeric gliadins on the other side act as plasticisers and therefore provide plasticity and viscosity to the dough (Khatkar et al., 1995; Goesaert et al., 2005). For bread making quality is measured mainly by bread volume, up to a certain point, high dough strength is favourable as it increases bread volume. Yet dough rise and therefore bread volume is negatively affected when the dough is too strong. It was found that differences in glutenin properties primarily affect gluten protein quality even though they are the smaller group considering their abundance. These differences can occur in the composition, structure and size distribution of glutenin polymers (Goesaert et al., 2005). Wheat varieties differ in the presence or absence of certain HMW-GS and LMW-GS, which explains the large variation in the composition of glutenin between wheat varieties (Gupta and Shepherd, 1990; Shewry et al., 1992). There are reports about positive correlations between dough strength and levels of large glutenin fractions, which led to the conclusion that only glutenin polymers above a certain size contribute to the elasticity of the glutenin polymer network (Singh and MacRitchie, 2001; Wieser, 2007). Due to the huge variation in gluten protein composition and the strong effects of small alterations, the relationship between wheat storage proteins and bread-making quality still needs further investigation.

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### 1.5. Objectives

Wheat is the second biggest staple crop worldwide and therefore is of great importance for human nutrition. As wheat is primarily consumed in form of baked goods, for wheat production the baking quality and thus the concentration and composition of storage proteins are of particular interest. Lately, for agriculture to be sustainable becomes more and more important and topics like sustainability, environmental protection and low nutrient input systems are being focussed in research. Nitrogen is one of the nutrients being under investigation due to its potential to unbalance ecosystems and to promote the greenhouse effect. With N being crucial for protein synthesis on one hand and critical for the environment on the other hand, well established fertilisation practices have to be questioned.

Even though the research of wheat proteins has a long history, the complex interactions between gluten protein composition and dough rheological properties are still not fully understood and were therefore addressed in this Ph.D. work. Furthermore, the effects of different N fertilisation regimes – especially a late N application – on storage protein composition, protein body distribution, gene expression of protein families and baking quality were evaluated.

#### **1.5.1. Splitting nitrogen applications improves wheat storage protein composition under low N supply (Chapter 2)**

The influence of split N application on gluten protein composition in winter wheat has barely been analysed. Following up the latest examinations of Xue et al., 2016, the effects of a split N application on the concentration and composition of wheat storage protein at different N fertilisation rates were investigated. The main focus was to test whether the effect of a split

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N application on the storage protein composition varies under different amounts of total N supply.

### **1.5.2. Foliar N application at anthesis alters grain protein composition and enhances baking quality in winter wheat only under a low N fertiliser regimen** (Chapter 3)

The foliar application of N fertiliser holds advantages, especially for the late time points in the growing season. By supplying N through the leaves, N losses due to denitrification and leaching can be reduced. Moreover, N can also be provided to the wheat plants in case of dry weather conditions (Gooding and Davies, 1992). The potential of a foliar N application at anthesis to affect gluten composition and baking quality was therefore evaluated.

### **1.5.3. Gene expression of grain protein fractions and protein body distribution in winter wheat as affected by foliar N application at anthesis** (Chapter 4)

In order to further understand the process of gluten protein synthesis and storage during grain development, the gene expression of gluten protein fractions was measured. Furthermore, the effects of a foliar N application at anthesis on the distribution of protein bodies within the wheat grain as well as the protein body size distribution were examined.

### **1.5.4. Effects of a late N fertiliser dose on storage protein composition and bread volume of two wheat varieties differing in quality** (Chapter 5)

New guidelines request a reduced input of N fertilisers into agricultural systems (Bundesministerium der Justiz und für Verbraucherschutz, 2017). Furthermore, the use of N fertiliser – particularly shortly before harvest – involves environmental risks, such as nitrate leaching and nitrous oxide emission (Senbayram et al., 2009). Thus, it was examined whether a reduced late N application via the soil is sufficient to produce wheat with a high baking

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quality. The effects of low and high late N application on gluten protein composition were also investigated.

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## Chapter 2

# Splitting nitrogen applications improves wheat storage protein composition under low N supply

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# Splitting nitrogen applications improves wheat storage protein composition under low N supply

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**Key words:** gliadin / glutenin / gluten protein / nitrogen fertilization

## 2. Split Nitrogen effects on storage protein composition

### **Abstract**

For the baking quality of wheat flours the composition and amount of grain protein are crucial. It is common practice to use late nitrogen (N) application to increase grain protein concentration (GPC) and hence, improve baking quality. However, the use of N fertilizer—particularly shortly before harvest—involves environmental risks. With the suitability of GPC as a parameter for baking quality predictions being more and more questioned, there are further investigations needed considering not only the GPC but also the composition of grain protein. Gluten protein composition varies depending on genotype and environmental factors, such as weather conditions and fertilization rate. To examine whether the effect of a split N application varies under different amounts of total N supply, this study investigates the effects of split nitrogen application on grain protein concentration and composition of wheat cultivar JB Asano at four different N fertilization levels (0.8; 1.0; 1.2 and 1.4 g N pot<sup>-1</sup>). The GPC was affected both, by N fertilization level and split N application. The minimum GPC of 13% which is required for class A wheat varieties, in this experiment was only achieved when N supply was moderate (at least 1.2 g N per pot). Considering the storage protein composition, the split N application influenced the proportion of  $\alpha$ -/ $\beta$ -gliadins and  $\gamma$ -gliadins, the alterations being inconsistent. The ratio of high molecular weight (HMW) to low molecular weight (LMW) glutenin subunits was increased by the split N application only at the lowest N fertilization level. It can be concluded that splitting N fertilization into three doses and hence, applying one dose of N late in the season can still be a useful approach to improve GPC as well as protein composition – especially when the total N supply is low.

## 2. Split Nitrogen effects on storage protein composition

### 1. Introduction

Wheat is one of the world's most important staple crops and is widely used to produce bread, pasta, and pastries. For baking quality, the composition and amount of grain protein are crucial. Along with Hagberg falling number and sedimentation value, grain protein concentration (GPC) is one main parameter to assess the baking quality and therefore to divide wheat genotypes into quality groups. In Germany there is a system with four wheat classifications: class E (elite wheat) consists of cultivars with excellent baking qualities; class A (quality wheat) is for cultivars with very good baking properties; cultivars with good baking qualities belong to class B (bread wheat); class C (miscellaneous wheat) contains mainly feed wheat varieties with low grain protein concentration which are not suitable for baking.

It is common practice to use late nitrogen (N) application to increase grain protein concentration and hence, improve dough rheological properties. The use of N fertilizer – particularly shortly before harvest – involves environmental risks, such as nitrate leaching and nitrous oxide emission (*Senbayram et al., 2009*). Hence, the beneficial effects of late nitrogen fertilizer use on storage protein composition must be investigated to evaluate the merits of split nitrogen fertilizer application.

Different storage proteins contribute to various rheological dough properties: monomeric gliadins are responsible for viscosity and extensibility of the dough, whilst polymeric glutenins give elasticity and strength (*Shewry and Tatham, 1997; Wieser, 2007*). Gluten protein composition varies depending on genotype and environmental factors, such as weather conditions and fertilization rate. *Schulz et al. (2015)* stated that it is not necessary to split N applications in wheat production but in their study, they only investigated the effects on grain protein concentration. With the suitability of this parameter for baking quality (bread volume) predictions being more and more questioned (*Kazman and Innemann, 2010*;

## 2. Split Nitrogen effects on storage protein composition

*Thanhaeuser et al., 2014; Gabriel et al., 2017*), there are further investigations needed, considering not only the GPC but also the composition of grain protein. In former investigations, a split N application was shown to improve baking quality due to alterations in grain protein composition (*Xue et al., 2016*). To examine whether the effect of a split N application varies under different amounts of total N supply, this study investigates the effects of split nitrogen application on wheat grain protein concentration and composition at different N fertilization levels.

## 2. Split Nitrogen effects on storage protein composition

### 2. Material and methods

#### 2.1 Plant cultivation and SPAD-measurements

A pot experiment was conducted in 2014/15 using the winter wheat variety JB Asano at four different nitrogen fertilizer levels (0.8, 1.0, 1.2, and 1.4 g N pot<sup>-1</sup>). Forty seeds were sown per pot, the number of plants being reduced to 14 plants per pot during early development. Plants were cultivated in Mitscherlich pots containing 6 kg of soil under natural conditions, with supplemental irrigation. Plants remained outside and were only moved to an unheated greenhouse for periods of heavy rain or frost. Nitrogen fertilization was split into either two or three applications [Growth stage (GS) 01, 31/32 and 49] applied as ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>). The amount of applied N for each of the two or three treatments was adjusted to maintain the same level of total applied N. This approach was required to isolate the effect of fertilizer timing from N rate effects. Five pot replicates were used for each nitrogen treatment. Before sowing, the following nutrients were provided to the soil in each pot: 0.6 g P, 2.3 g K, 1.2 g Ca, 0.5 g S, 0.3 g Mg, 0.010 g Cu, 0.015 g Zn, and 0.030 g Mn.

SPAD-values were measured on flag leaves as soon as these were fully developed and until they were completely senescent. The average of ten measurements with a chlorophyll meter (SPAD-502, Konica Minolta Sensing Europe B.V.) per pot was determined.

#### 2.2 Yield, N and protein concentration

Whole plants were harvested, and grain, chaff, flag leaf, and straw were separated. The dry weight of each fraction and thousand grain weight (TGW) were recorded. All plant fractions were ground and nitrogen concentration was determined using a CNS analyser (Flash EA 1112 NCS, Thermo Fisher Scientific, Waltham, MA, USA). Grain protein concentration was calculated by multiplying N concentration by 5.7.

## 2. Split Nitrogen effects on storage protein composition

### 2.3 Protein extraction and SDS-PAGE

Storage protein fractions were extracted stepwise from 100 mg wholemeal flour according to Xue et al. (2016). After extracting the fraction of albumins and globulins by using extraction buffer (0.4 M L<sup>-1</sup> NaCl, 67 mM L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 7.7 mM L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, pH 7.6), the fraction of gliadins was extracted by 60% ethanol. The remaining pellet was then washed with deionised water and the fraction of glutenins was obtained by extraction with extraction buffer [50% 1-propanol (v/v), 2 M L<sup>-1</sup> urea, 1% dithiothreitol (DDT), 0.05 M L<sup>-1</sup> Tris-HCl, pH 7.5]. An aliquot of each sample (300 µL) was transferred into a new tube and the contained protein was precipitated by adding 900 µL DDT/acetone solution (50 mM L<sup>-1</sup> DDT) and storing the samples at -20°C overnight. After centrifugation (16000 g, 25 min, 4°C), the supernatant was discarded and the protein pellet was dissolved in lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 30 mM DTT, 20mM Tris base), subsequently, samples were stored at -20°C. The 2-D Quant Kit (GE Healthcare Life Science, Uppsala, Sweden) was used to quantify the extracted protein. SDS-PAGE was performed as per the method of *Laemmli* (1970) with some modifications to determine gliadin and glutenin composition in wheat grain. Medium sized gels (180 × 160 × 1.5 mm) consisting of 12.5% separating gel and 6.3% stacking gel were prepared one day prior to use. An equal amount of 20 µg of protein was combined with loading buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 200 mM DTT] and was then applied to each well. Roti®-Mark 10–150 (Carl Roth GmbH + Co. KG, Karlsruhe) was applied to each gel as protein ladder. Gels were run for 5 hours at 250 V. After completion of the run, gels were fixed in a fixing solution (40% ethanol, 50% H<sub>2</sub>O, and 10% acetic acid) for one hour. Afterwards, gels were stained with staining solution [1 Coomassie tablet (GE Healthcare) in 1.6 L 10% acetic acid] for 1 h and later, destained in 10%

## 2. Split Nitrogen effects on storage protein composition

acetic acid overnight. Gels were then scanned using an Epson Perfection V700 scanner. Gel image analyses were carried out using the GelAnalyzer 2010a software ([gelanalyzer.com](http://gelanalyzer.com)).

### **2.4 Statistical analysis**

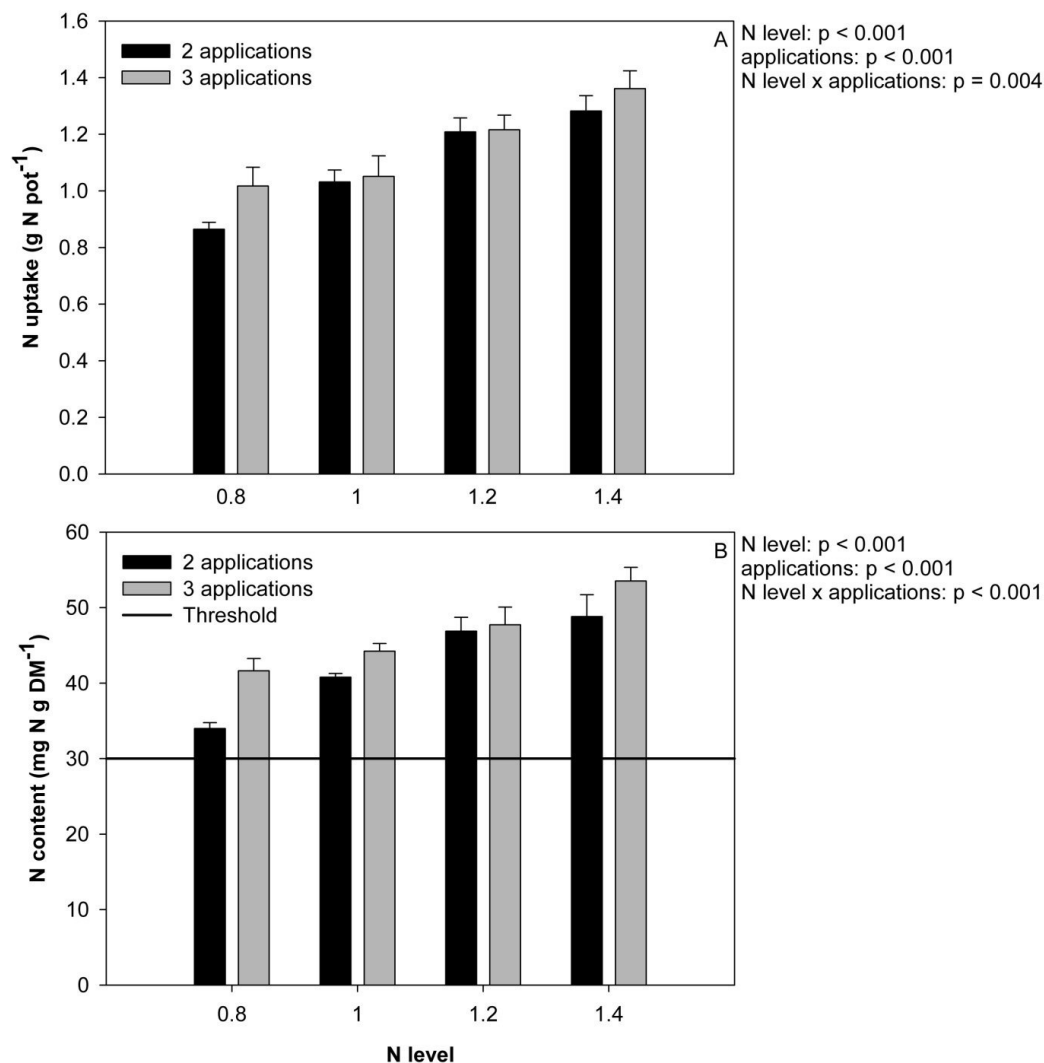
The GenStat (17<sup>th</sup> edition, VSN International Ltd., Hemel Hempstead, UK) software was used for statistical analyses. Data were analysed by ANOVA, the design structure of the experiment (randomised pots) being taken into account. Regression analysis was used to evaluate relationships between various storage protein fractions and GPC.



## 2. Split Nitrogen effects on storage protein composition

### 3. Results

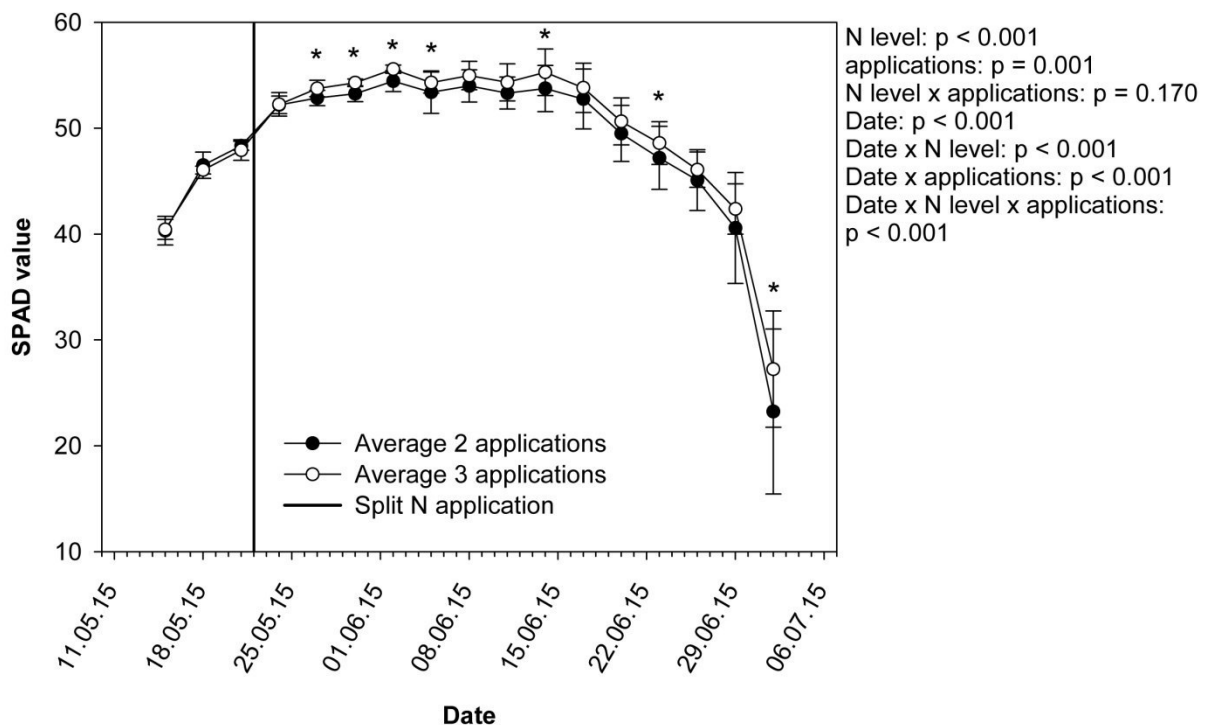
The plant N uptake increased accordingly to the N fertilization levels with N uptake being the lowest at the N level of 0.8 g N and the highest at the N level of 1.4 g N (Fig. 1A). The splitting of N fertilization into 3 applications enhanced plant N uptake only in the lowest and highest N level (0.8 and 1.4 g N). The plant N content ranged from 34.0 to 53.5 mg N g dry matter (DM)<sup>-1</sup> and was increased by the splitting of the N applications in all N fertilization levels except the N level of 1.2 g N. With increasing amounts of N supplied, the plant N content increased (Fig. 1B).



**Figure 1:** Plant N uptake (A) and plant N content (B) of wheat cultivar JB Asano at four N levels (0.8, 1.0, 1.2, 1.4 g N pot<sup>-1</sup>) with two or three N applications.

## 2. Split Nitrogen effects on storage protein composition

SPAD-values were measured on flag leaves as soon as these were fully developed and until they were completely senescent. Significant differences between the two versus three N applications were monitored from five days after the third N application (Fig. 2) with higher SPAD-values for the variants which received a split N application. In general, SPAD-values increased until reaching a maximum of 55.0 on 02.06.2015. There was a steady decline of SPAD-values observed after June 14, 2015, with flag leaves being fully senescent after July 02, 2015.



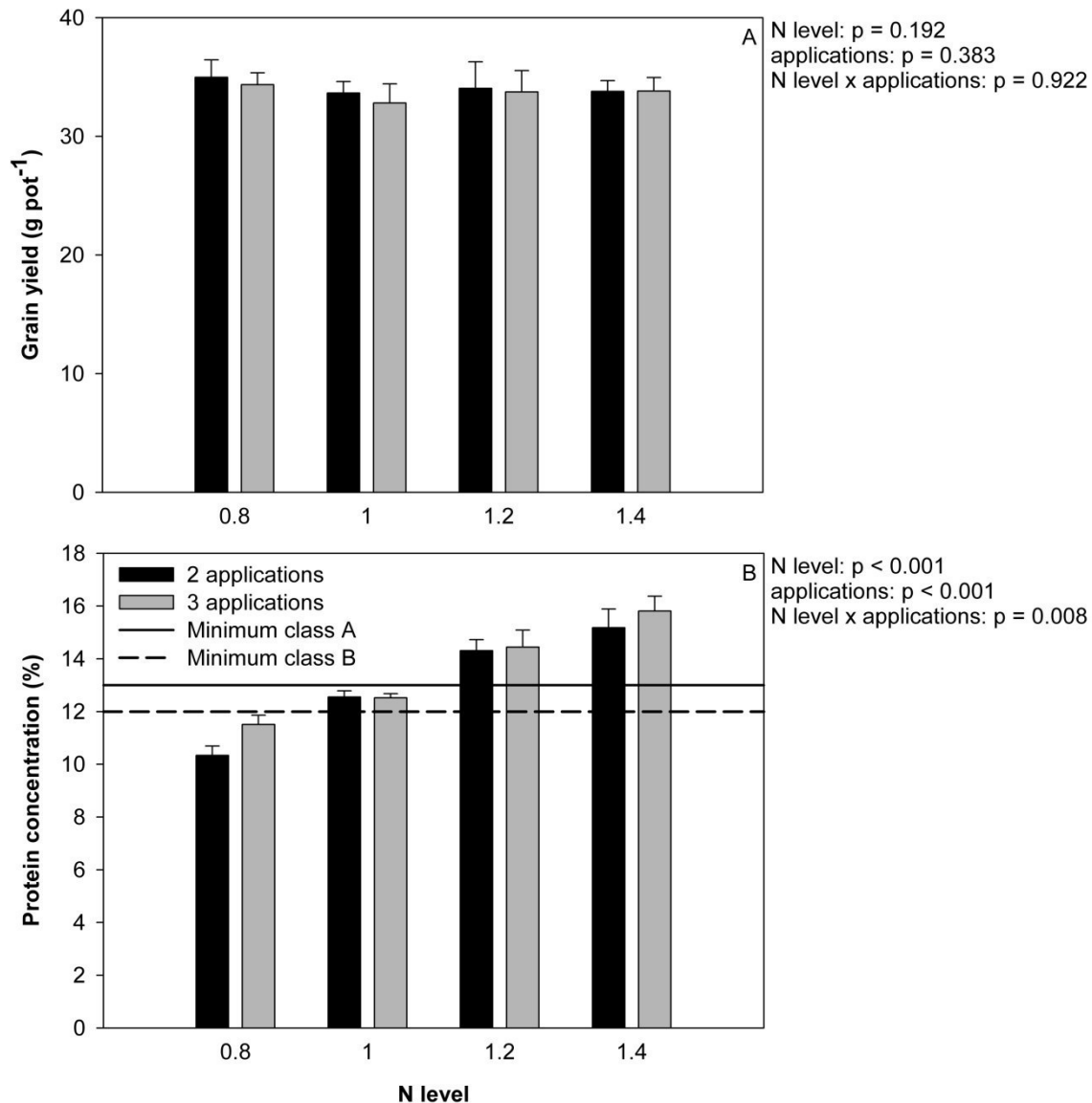
**Figure 2:** SPAD values of wheat cultivar JB Asano during grain development

Grain yield of the wheat plants was neither affected by N fertilization level nor by the split N application. The grand mean of grain yield of all treatments was  $33.9 \text{ g pot}^{-1}$ .

The grain protein concentration determines the market value of harvested wheat and is used as a parameter for baking quality. With increasing N fertilization level the grain protein concentration was enhanced (Fig. 3B). A minimum grain protein concentration of 10.3 % was

## 2. Split Nitrogen effects on storage protein composition

achieved at 0.8 g N without split N application and the highest grain protein concentration with 15.8 % was obtained at 1.4 g N with split N application. The splitting of N fertilization into three applications improved grain protein concentration only at the lowest and the highest N fertilization level (0.8 g N and 1.4 g N). A minimum of 13 % grain protein concentration required for class A wheat was only obtained with at least 1.2 g N supply.



**Figure 3:** Grain yield (A) and grain protein concentration (B) of wheat cultivar JB Asano at four N levels (0.8, 1.0, 1.2, 1.4 g N pot<sup>-1</sup>) with two or three N applications.

## 2. Split Nitrogen effects on storage protein composition

Harvest index (HI) is a measure of productivity of plants and was calculated by using the following equation:

$$HI [\%] = \frac{\text{Grain yield}}{(\text{Grain yield} + \text{Straw biomass})} \times 100. \quad (1)$$

As shown in Fig. 4A, the harvest index was only affected by the N fertilization level but not by split N application. The harvest Index, with 71.5 %, was significantly higher at the 0.8 g N level compared to the remaining three N fertilization levels (1.0 g N: 66.7 %; 1.2 g N: 65.1 %; 1.4 g N: 63.7 %).

Nitrogen harvest index (N-HI) was calculated according to the following equation:

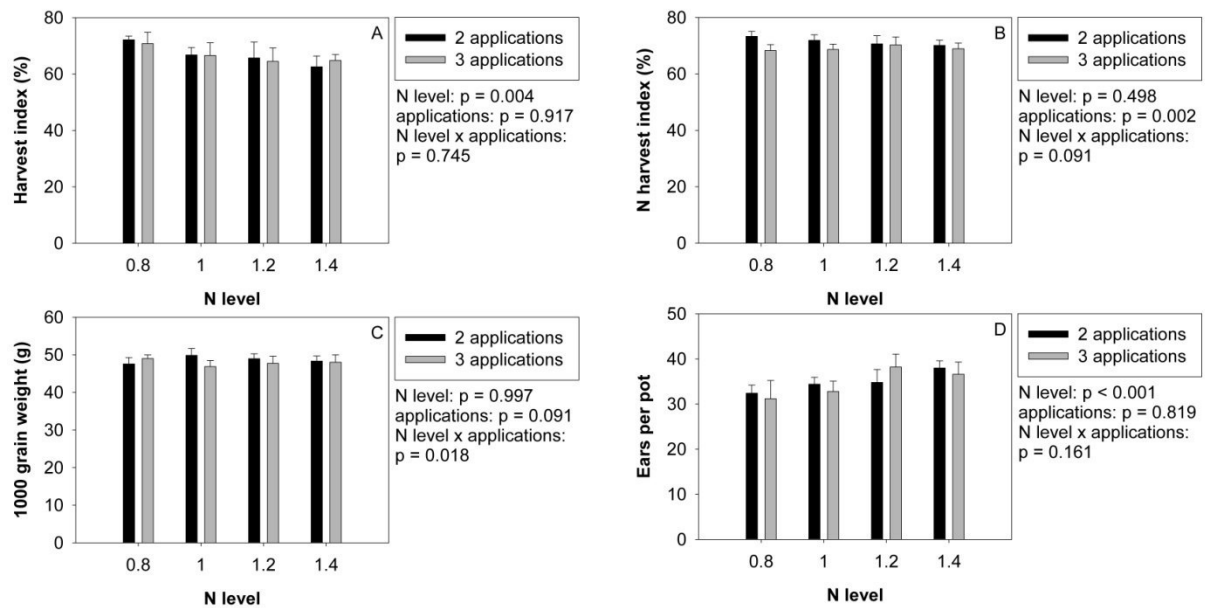
$$NHI [\%] = \frac{\text{Grain N content}}{\text{Shoot N content}} \times 100. \quad (2)$$

The N fertilization level did not alter the NHI but when the same amount of N was split into three instead of two applications, the NHI decreased from 71.5 % to 69.0 % (Fig. 4B).

Thousand grain weight (TGW) is a measure of grain size and was decreased by split N application only at the 1.0 g N level (Fig. 4C). When supplied N was split into three applications TGW was the same for all tested N levels but when N was supplied in only two applications TGW at 1.0 g N was higher than at 0.8 g N.

The number of ears per pot was increased with increasing N fertilization rate, with a significant difference between 1.0 g N (33.6 ears per pot) and 1.2 as well as 1.4 g N (36.5 and 37.3 ears per pot, respectively) being observed (Fig. 4D). The split N application did not affect the number of ears per pot.

## 2. Split Nitrogen effects on storage protein composition

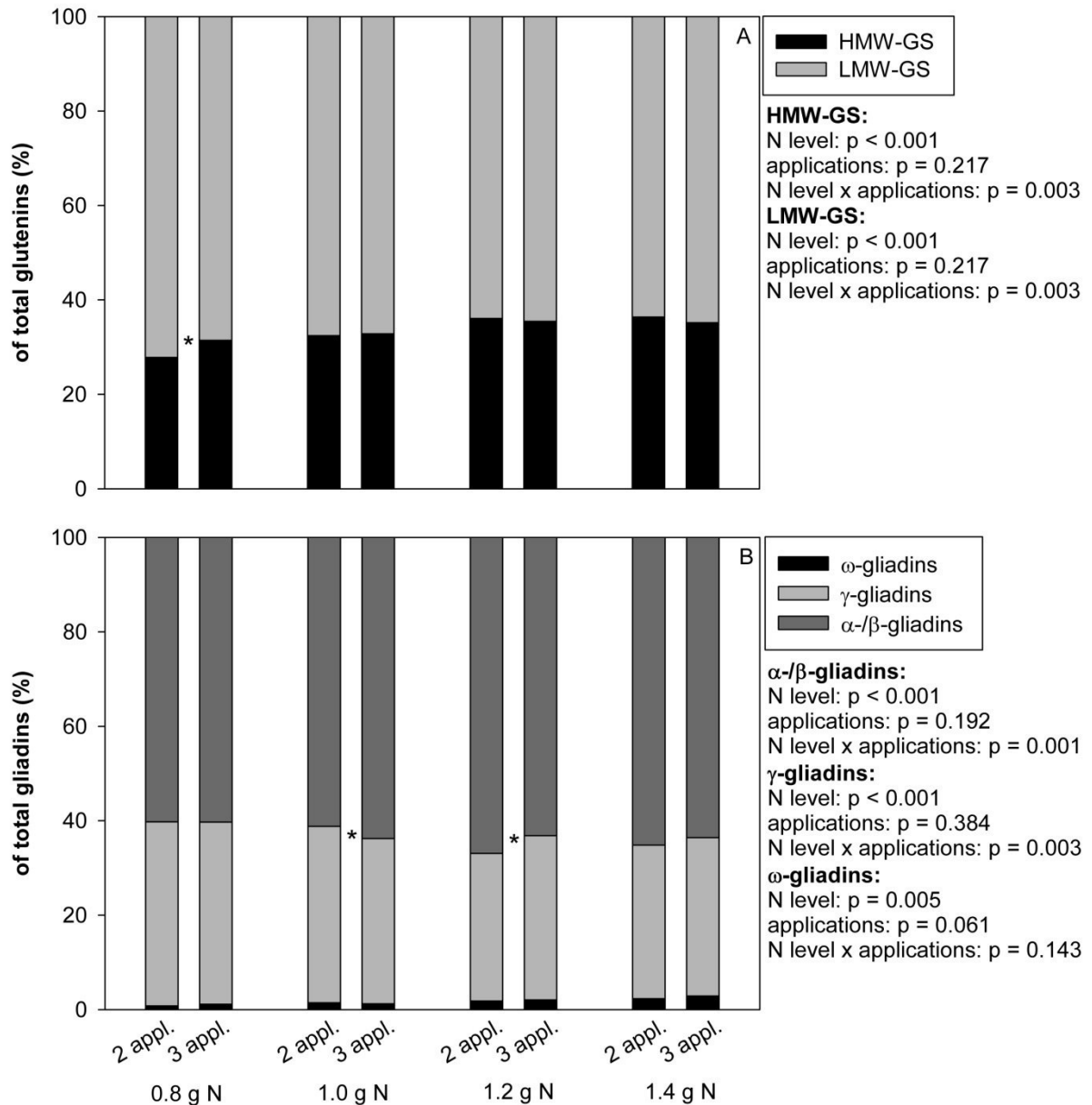


**Figure 4:** Harvest index (A), N harvest index (B), 1000 grain weight (C) and ears per pot (D) of wheat cultivar JB Asano at four N levels (0.8, 1.0, 1.2, 1.4 g N pot<sup>-1</sup>) with two or three N applications.

From the flour, the gliadin and glutenin fractions were extracted and divided by size using SDS-PAGE. The glutenins were separated into their high molecular weight (HMW-GS) and low molecular weight (LMW-GS) subunits. With a proportion of 65–72 %, the latter was the major glutenin subunit (Fig. 5A). The N fertilization level did not affect the concentrations of HMW-GS and LMW-GS of total glutenins. While split N application increased the proportion of HMW-GS at the lowest N fertilization level (0.8 g N), the proportion of LMW-GS was decreased. The gliadin fraction was divided into  $\alpha$ -/ $\beta$ -gliadins,  $\gamma$ -gliadins and  $\omega$ -gliadins; their proportions are shown in Fig. 5B. The  $\alpha$ -/ $\beta$ -gliadins are the biggest group with a proportion of 61–67 % of total gliadins, followed by the  $\gamma$ -gliadins with a proportion of 31–39 %. The  $\omega$ -gliadins are the group with the least proportion (1–3 %). The proportion of  $\omega$ -gliadins increases slightly due to increased N supply, with the proportions being significantly different between 0.8 g N and 1.2 g N as well as 1.4 g N and between 1.0 g N and 1.4 g N.

## 2. Split Nitrogen effects on storage protein composition

The split N treatment only affected the proportions of  $\alpha$ -/ $\beta$ -gliadins and  $\gamma$ -gliadins at the N levels of 1.0 and 1.2 g N. At 1.0 g N the split N application increased the proportion of  $\alpha$ -/ $\beta$ -gliadins and simultaneously decreased the proportion of  $\gamma$ -gliadins. At the N fertilization level of 1.2 g N, this effect was reversed.

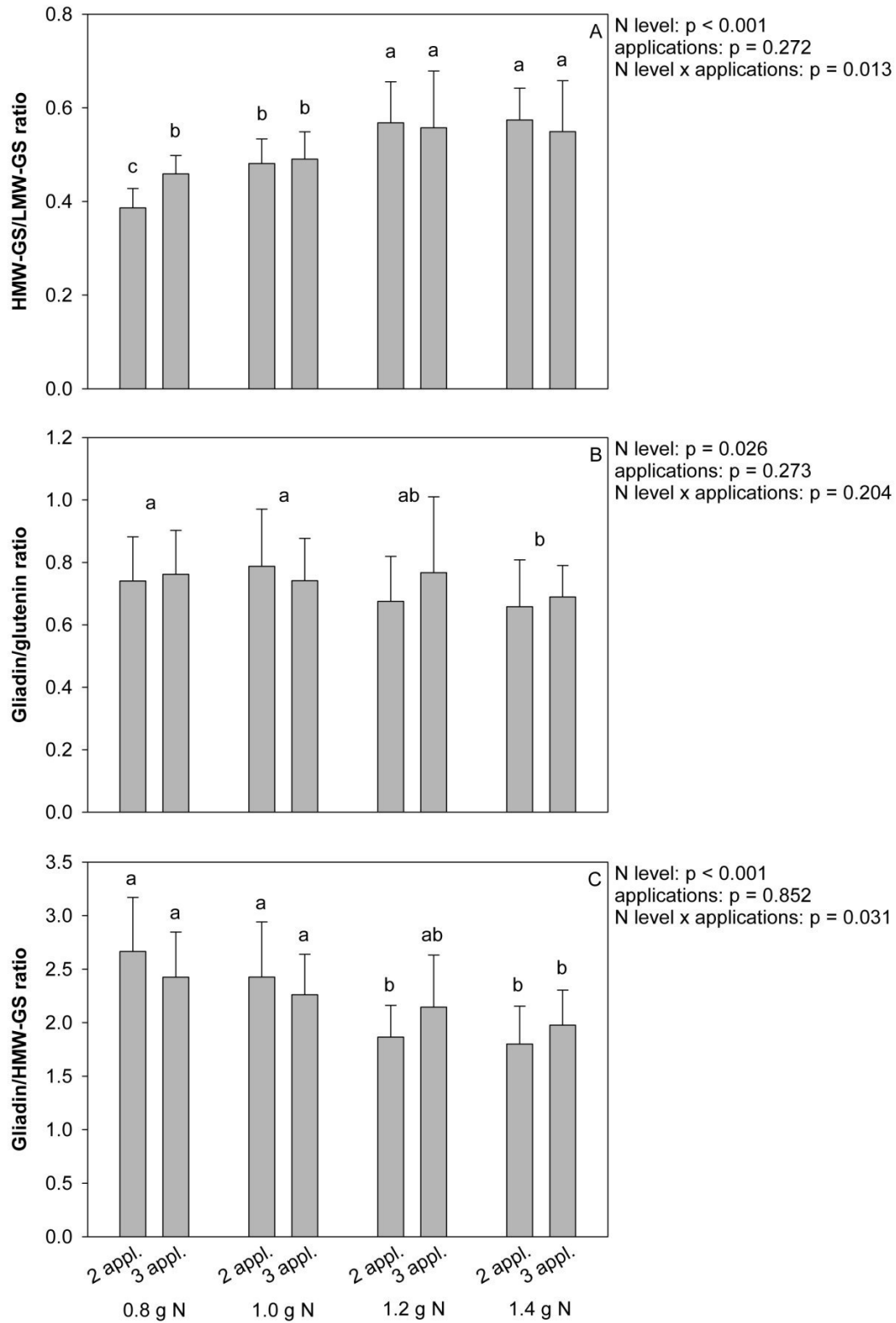


**Figure 5:** Composition of glutenin subunits (A) and gliadin fractions (B) of wheat cultivar JB Asano at four N levels (0.8, 1.0, 1.2, 1.4 g N pot<sup>-1</sup>) with two or three N applications.

## 2. Split Nitrogen effects on storage protein composition

Ratios between certain storage protein fractions, such as HMW-GS/LMW-GS ratio, gliadin/glutenin ratio and gliadin/HMW-GS ratio, are used as indicators for the baking quality of the flour (Godfrey et al., 2010; Millar, 2003). The HMW-GS/LMW-GS ratio was increased by the split application of N only at the lowest N fertilization level (Fig. 6A). With increasing N fertilization level, the ratio of HMW-GS/LMW-GS increased slightly. The gliadin/glutenin ratio was not altered by the split application of N but was slightly decreased by increasing N fertilization level with significant differences being observed between 1.4 g N and 1.0 g N as well as 0.8 g N (Fig. 6B). The split N application did not affect the gliadin/HMW-GS ratio but with increasing N fertilization level a slight decrease of this ratio was observed (Fig. 6C). Significant differences occurred between the two lowest (0.8 and 1.0 g N) and the two highest (1.2 and 1.4 g N) N fertilizer levels when N was supplied in two applications. When N fertilization was split into three applications, the gliadin/HMW-GS ratio at 1.4 g N was significantly lower than at the N levels of 0.8 and 1.0 g N.

## 2. Split Nitrogen effects on storage protein composition



**Figure 6:** Ratios of HMW-GS/LMW-GS (A), gliadin/glutenin (B) and gliadin/HMW-GS (C) of wheat cultivar JB Asano at four N levels (0.8, 1.0, 1.2, 1.4 g N pot<sup>-1</sup>) with two or three N applications.



## 2. Split Nitrogen effects on storage protein composition

### 4. Discussion

The grain yield remained unaffected by both N fertilization level and split N application. This was not unexpected because with the splitting of N applications no additional amount of N was applied to the plants and, therefore, no effect of split N application on grain yield was predicted. For the steps of the N fertilization levels were chosen very small, even additional N amounts did not alter grain yield. As the main goal of this study was to investigate the effects of split N application on grain protein concentration and composition at different N fertilization levels, no differences in grain yield were intended.

The HI was decreased only by the level of supplied N; this means that with increasing N supply the straw biomass was enlarged with the grain yield remaining stable. Increasing N fertilization is reported to have varying effects on HI (positive, negative or no effects at all) (Pask et al., 2012). With a decrease from 71.5 % at the lowest N level (0.8 g N) to 63.7 % at the highest N level (1.4 g N) the modification was modest. The N-HI was decreased by split N application, which could possibly be explained by reduced N remobilisation from shoot to grain caused by soil N being available due to the split N application. This goes along with reports on a negative correlation between remobilisation of N from vegetative organs and post-anthesis N uptake from the soil (Bogard et al., 2010; Taulemesse et al., 2015). The likely reduction of N remobilisation from leaves into the grain is supported by the positive effect of the split N application on chlorophyll content in flag leaves (SPAD-values) during the grain filling period, as leaf senescence is associated with N translocation to the grains (Bogard et al., 2011).

The GPC was affected both by N fertilization level and by split N application. Every step of additional N led to an increase in GPC. At the highest N fertilization level (1.4 g N) the GPC was 4.6 % higher than at the lowest N fertilization level (0.8 g N), even though the amount of

## 2. Split Nitrogen effects on storage protein composition

additional N was rather small. The variety JB Asano that was used in this experiment belongs to the class A according to the German Federal Plant Variety Office (*Bundessortenamt*, 2017). For wheat varieties from this classification, a minimum GPC of 13 % is required, which in this experiment was only achieved when at least 1.2 g N per pot were applied. The minimum of 12 % required for class B was achieved when at least 1.0 g N per pot were applied. Due to split N application, the GPC was increased at the lowest (0.8 g N) as well as the highest (1.4 g N) N fertilization level with the increase by 1.2 % at the lowest N level being greater than at the highest N level (0.6 %).

The extracted glutenins were separated into HMW-GS and LMW-GS and their proportions of the total glutenin fraction were calculated. An interaction of N fertilization level and split N application was observed for both glutenin fractions. The only impact of the split N application occurred at the lowest N fertilization level (0.8 g N), with the proportion of HMW-GS being increased while the proportion of LMW-GS decreased due to the split N application. The slight increase of HMW-GS and decrease of LMW-GS with increasing N fertilization levels indicates a connection between the proportions of the two glutenin fractions and the GPC. This connection was tested using regression analysis (data not shown). With a correlation coefficient of 0.266, there was no correlation observed between the subunits of glutenins and the GPC. The gliadins were divided by size into the following fractions:  $\alpha$ -/ $\beta$ -gliadins,  $\gamma$ -gliadins and  $\omega$ -gliadins. All gliadin fractions were affected by the level of N fertilization, with proportions of  $\alpha$ -/ $\beta$ -gliadins and  $\omega$ -gliadins being enhanced with increasing levels of N fertilization while proportions of  $\gamma$ -gliadins were diminished with increasing N fertilization level. These results go along with findings from *Daniel and Triboí* (2002) and *Hurkman et al.* (2013), who also showed that proportions of those proteins with a low or medium S content (such as  $\omega$ -gliadins and HMW-GS) increase while proportions of

## 2. Split Nitrogen effects on storage protein composition

S-rich proteins (such as LMW-GS) decrease with increasing N fertilization level. The split N application only influenced the proportion of  $\alpha$ -/ $\beta$ -gliadins and  $\gamma$ -gliadins with the alterations being inconsistent. While the proportions of these gliadin fractions were not altered at the lowest and highest N fertilization level, for  $\alpha$ -/ $\beta$ -gliadins an increase due to split N application was observed at 1.0 g N and a decrease at 1.2 g N. For  $\gamma$ -gliadins these effects were reversed. *Lindeque et al. (2017)* also found, that the relationships between glutenin, gliadin, and albumin/globulin fractions and GPC were not consistent.

The ratios of certain protein fractions or subunits (such as HMW-GS/LMW-GS, gliadin/glutenin, and gliadin/HMW-GS) strongly contribute to the baking performance of the flour (*Millar, 2003; He et al., 2013; Thanhaeuser et al., 2014*). Therefore it is of interest to investigate the influence of split N application not only on the different protein fractions and subunits but also on their ratios. While the level of N fertilization affected all three ratios analysed (HMW-GS/LMW-GS, gliadin/glutenin, and gliadin/HMW-GS) the split N application influenced the HMW-GS/LMW-GS ratio alone (Fig. 6). The ratio of HMW-GS to LMW-GS was increased significantly at the lowest N fertilization level (0.8 g N) due to split N application. As GPC was increased by split N application at the lowest as well as the highest N level, it can be concluded that the composition of glutenin subunits is independent of total protein concentration in the grain. As *Thanhaeuser et al. (2014)* discovered an improved bread volume as a consequence of decreased LMW-GS/HMW-GS ratio (which means increased HMW-GS/LMW-GS ratio) it can be assumed that the enhanced ratio of HMW-GS to LMW-GS due to split N application in this experiment would lead to improvements in baking performance. The ratio of gliadins to glutenins was decreased in the highest N fertilization level (1.4 g N) compared to the two lowest N levels (0.8 and 1.0 g N). This finding is comparable with results from *Bonnot et al. (2017)* that show an increased ratio of gliadins to

## 2. Split Nitrogen effects on storage protein composition

glutenins when the grain N/S ratio decreased. As a result of increasing amounts of N fertilizer in this experiment, the N/S ratio is likely to increase as well because the S supply remained unchanged. This increase in grain N/S ratio leads to a decreased ratio of gliadins to glutenins.

In this controlled pot experiment under natural conditions, neither the proportions of glutenin subunits nor the proportions of gliadin fractions were well correlated to GPC. As differences in the composition of these storage protein fractions have been found to be connected to changes in baking performance (*Wieser and Kieffer, 2001; Thanhaeuser et al., 2014; Lindeque et al., 2017*) this underlines the necessity of a combined analysis of GPC and storage protein composition in order to evaluate wheat baking properties.

## 2. Split Nitrogen effects on storage protein composition

### 5. Conclusions

The GPC was altered both by N fertilization level and by split N application while grain yield remained unaffected. The minimum GPC of 13 % which is required for class A wheat varieties, in this experiment was only achieved when a moderate amount of N (at least 1.2 g N per pot) was applied. The split N application improved GPC only at the lowest and the highest N fertilization level. While the positive effect of splitting N fertilization into three doses on GPC at the low N level is plausible, the reasons for the effect at the high N level are not clear. Considering the storage protein composition, the split N application only influenced the proportion of  $\alpha$ -/ $\beta$ -gliadins and  $\gamma$ -gliadins on the one hand with the alterations being inconsistent. On the other hand the ratio of HMW-GS to LMW-GS was only increased by the split N application at the lowest N fertilization level. These changes in the composition of storage proteins are known to positively impact the bread making performance. In general, the effects of split N application were more frequent when total N supply was low. Therefore it can be concluded that splitting N fertilization into three doses and hence, applying one dose of N late in the season (around anthesis) can still be a useful method to improve GPC as well as protein composition – especially when the total amount of N fertilizer is rather low.

### 6. Acknowledgments

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## Chapter 3

# Foliar N application at anthesis alters grain protein composition and enhances baking quality in winter wheat only under a low N fertiliser regimen

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Mühling, K. H.*

submitted (24<sup>th</sup> July 2018)

# Foliar N application at anthesis alters grain protein composition and enhances baking quality in winter wheat only under a low N fertiliser regimen

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## **Highlights:**

- Increasing protein concentration does not necessarily mean better baking quality
- Baking performance depends on composition of storage proteins
- Late foliar N application improves baking quality when N supply is low
- For baking quality evaluation, protein composition and bread volume are required

Declaration of interest: none

### 3. Foliar N application effects on grain protein composition and baking quality

#### **Abstract**

Wheat is the second biggest staple crop worldwide and is mainly consumed in the form of baked goods, requiring a specific flour quality. Grain protein concentration (GPC) is an underpinning parameter for baking quality and therefore strongly influences the value of wheat. It is a common strategy to increase GPC by combining high protein varieties with the application of a late dose of nitrogen. However, the late use of N fertilisers can cause environmental problems, such as nitrate leaching and gaseous losses. Furthermore, recent studies show, that there is only a weak relationship between GPC and bread volume. The aim of this study was to re-evaluate the merits of a late N application by investigating the effects of a late foliar N application, not only on GPC, but also on the gluten protein composition and on bread volume.

In this study, an increasing GPC did not necessarily lead to improved baking quality. Baking performance rather depended on the grain protein composition. Only at a low N fertiliser level (100 kg N ha<sup>-1</sup>), the foliar N application decreased the HMW-GS/LMW-GS ratio and increased the gliadin/HMW-GS ratio, which led to an improved bread volume. These results imply that a late foliar N application can be used to effectively improve baking quality when the total N uptake was low due to low fertilisation or unfavourable weather conditions. The results also show that quality cannot be evaluated by measuring GPC alone but also needs information of storage protein composition as well as bread volume.

**Key words:** nitrogen, wheat, gliadin, glutenin, bread volume

**Abbreviations:** GPC, Grain protein concentration; HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits; TGW, 1000 grain weight; HFN, Hagberg falling number; NABIM, National Association of British and Irish Millers

### 3. Foliar N application effects on grain protein composition and baking quality

#### 1. Introduction

With a worldwide production of 749 million tonnes in 2016, wheat (*Triticum aestivum* L.) is the second biggest staple crop worldwide. After rice, it is the most important food for humans, and by providing 16 g protein capita<sup>-1</sup> day<sup>-1</sup> (world average), it is the main source of protein in human nutrition (<http://www.fao.org/faostat/en/#compare>). Wheat products are mainly baked goods, such as bread, buns, pizza or pastries, and a high baking quality of wheat flour is required. Grain protein concentration (GPC) is an essential parameter for baking quality predictions and therefore often determines the price for wheat grain. In the UK, wheat cultivars are classified into quality groups (NABIM (National Association of British and Irish Millers) groups 1 to 4) using GPC as one of the main parameters. As wheat quality characteristics are defined by genotype, environment and their interaction, it is a well-known strategy for farmers to increase GPC by combining high protein varieties with the application of a late dose of nitrogen fertiliser. However, recent studies show that there is only a weak relationship between GPC and bread volume, which is a direct measure of baking quality (Kazman and Innemann, 2010; Thanhaeuser et al., 2014). Especially for high protein wheat cultivars with GPCs greater than 12 %, only a poor correlation was observed (Gabriel et al., 2017). At present, no better (quickly detectable) parameter other than GPC has been found. The ideal approach would be to combine GPC measurements with the examination of the composition of grain proteins as well as baking performance of the flours to evaluate the end-use quality of wheat grains.

The late use of N fertilisers can cause environmental problems, such as nitrate leaching into the groundwater and gaseous losses in the form of nitrous oxide, which contribute to global warming (Senbayram et al., 2009). For farmers, these problems represent a monetary risk, particular in the case of unfavourable weather conditions which may limit the uptake of the

### 3. Foliar N application effects on grain protein composition and baking quality

supplied nitrogen and hence, the required GPC might not be reached. In this case, a large amount of N remains in the soil and increases the environmental risks mentioned above. Effects of N fertiliser management on GPC, grain protein composition and baking quality have been investigated previously (Pechanek et al., 1997; Wieser and Seilmeier, 1998; Wan et al., 2014; Schulz et al., 2015; Xue et al., 2016a; Xue et al., 2016b), but the observed changes in protein composition due to different N fertilisation managements and their influence on baking quality are inconsistent. Furthermore, the influence of a foliar N application was considered in none of these studies. Woolfolk et al. (2002), Bly and Woodard (2003) and Tea et al. (2004) studied the influence of a foliar N application on various quality parameters, but not on bread volume. For example Wieser and Kieffer (2001) found a strong influence of the gliadin/glutenin ratio on rheological properties and bread volume, but this could not be confirmed by Pechanek et al. (1997). Another example is the influence of N fertilisation on the quantity of HMW-GS, which was reported to be positive (Wieser and Seilmeier, 1998) and negative (Pechanek et al., 1997). As the relationship of protein composition and baking quality remains unclear, further investigations are needed.

In this context, the aim of this study was to re-evaluate the merits of a late N application by investigating not only the effects of a late foliar N application on GPC, but also on the composition of grain proteins and on the bread volume, in three wheat cultivars, under two N fertiliser regimes.

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## 2. Material and Methods

### 2.1. Plant cultivation

Two bread-making wheat cultivars (Skyfall and Soissons) and one feed wheat cultivar (Conqueror) were grown in a randomised field experiment, with three replicate blocks at Rothamsted Research (Harpenden, UK) in 2016 and 2017, as part of the Defra-sponsored Wheat Genetic Improvement (WGIN) trials (Barraclough et al., 2010). Nitrogen was applied at two levels, 100 kg N ha<sup>-1</sup> (N100) and 200 kg N ha<sup>-1</sup> (N200) and an additional late foliar application of urea (40 kg N ha<sup>-1</sup>) at anthesis (growth stage (GS) 69) was performed on one half of each plot, resulting in the following four treatments: 100 kg N ha<sup>-1</sup>; 100 kg N ha<sup>-1</sup> + foliar N; 200 kg N ha<sup>-1</sup>; 200 kg N ha<sup>-1</sup> + foliar N. Nitrogen was applied as ammonium nitrate at two time points for N100 (50-50-0) and at three time points for N200 (50-100-50) during vegetative plant growth (respectively, approximately GS 24, 31 and 32). In 2016, ears were tagged at anthesis and caryopses were harvested from the middle of the ear at 10, 14, 21, 28 and 35 days post-anthesis (DPA) and immediately frozen in liquid nitrogen for protein extraction. Cross sections of the caryopses were taken and fixed (4% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer, pH 7.4) at each time-point for microscopic analysis. After anthesis, weekly measurements with the HandySpec® Systems (Tec5 AG, Germany) and SPAD meter (SPAD-502, Konica Minolta Sensing Europe B.V.) were carried out to monitor senescence. Plant protection was carried out according to local farming practice. Weather data for the two years of field experiments were obtained from the Rothamsted meteorological station (<http://resources.rothamsted.ac.uk/environmental-change-network/rothamsted-weather-charts>).

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#### 2.2. Yield, N, GPC and N-harvest index

Plants were harvested from a sample area (0.5 m<sup>2</sup>) in each plot at anthesis and at maturity. Mature plants were threshed and grain and straw fresh and dry weight were recorded as well as thousand-grain-weight (TGW) and Hagberg Falling number (FN). Yields are expressed as 85 % dry matter (DM). The weight of flour used for Hagberg Falling Number measurement was adjusted according to the moisture content of the flour. Grain and straw samples were ground with an Ultra Centrifugal Mill (ZM 200, Retsch) and a Hammer Mill (Christy & Norris 8" Lab Mill, Christy Turner Ltd) for further analysis. The nitrogen content of grain and straw samples was measured by the Dumas method using a LECO CN628 Combustion Analyser (LECO Corporation, St Joseph, Michigan, USA) and is expressed in percent of dry matter. Grain protein concentration was calculated by multiplying the N concentration by the factor 5.7. Elemental analyses of grain and straw samples were carried out using ICP-MS (inductively coupled mass spectrometry). Nitrogen harvest index (N-HI) was calculated according to the following equation:

$$NHI [\%] = \frac{\text{Grain N content}}{\text{Shoot N content}} * 100$$

#### 2.3. Micro baking test

The moisture content of flour samples was calculated from the weights of a given amount of flour before and after drying for 2 hours at 110 °C. Afterwards, 10 g of wholemeal flour (14.0 % moisture) were mixed with 0.2 g NaCl and then used to determine optimal water uptake and optimal dough kneading time by means of a farinograph (Farinograph-E, Brabender GmbH & Co. KG, Duisburg, Germany). An optimal dough development was defined at a dough consistency of 550 Brabender Units (530-570 BU) (Kieffer et al., 1998). For the microscale baking test, 10 g of flour (14.0 % moisture) were combined with 0.7 g fresh yeast

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and 0.1 g shortening. 1 mL NaCl/sucrose-solution (1 % NaCl, 2 % sucrose), 0.3 mL 0.004 M L(+)-ascorbic acid solution and the determined amount of water were added and the dough was kneaded for the optimal time required to reach 550 BU. After kneading, the dough was left to rest at 30 °C and 90 % relative humidity for 20 minutes. The dough was reshaped and rounded before secondary proofing for 40 minutes. Finally, the dough was baked for 10 minutes at 180 °C increasing to 250 °C on an automated proofing baking line. The bread loaves were weighed after cooling and bread volume was measured by surface scanning, using a Volscan Profiler 300 (Stable Micro Systems Ltd, Godalming, UK).

#### 2.4. Protein extraction and SDS-PAGE

Grain samples taken at five time-points after anthesis (10, 14, 21, 28, 35 DPA) were ground using a freezer mill (Freezer Mill 6870, Spex Sample Prep). Aliquots of each sample were freeze-dried for at least 36 hours. Total gluten protein fraction was extracted from 10 mg flour using 50% propan-1-ol + 2% dithiothreitol (DTT) at 50 °C, the extraction being repeated and the supernatants combined. After freeze-drying, the protein was dissolved in lysis buffer (8 M urea, 2 M thiourea, 4 % CHAPS, 30 mM DTT, 20mM Tris base). The 2-D Quant Kit (GE Healthcare) was used to quantify the extracted protein. The sample amount containing 10 µg protein was combined with loading buffer (50 mM Tris-HCl (pH 6.8), 2 % SDS, 10 % glycerol, 0.1 % bromophenol blue, 200 mM DTT) and loaded on 7 cm precast gels (4-15% Mini-PROTEAN TGX Precast Protein Gels, Biorad, Munich, Germany) for 1D SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Precision Plus Protein™ Standard (Biorad) was applied to each gel as protein ladder. Running conditions were 40 V for 30 minutes, then 80 V for 120 minutes. After completion of the run, gels were fixed in a fixing solution (40 % ethanol, 50 % H<sub>2</sub>O and 10 % acetic acid) for one hour. Afterwards, gels



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were stained with staining solution (1 Coomassie tablet (GE Healthcare, Freiburg, Germany) in 1.6 L 10 % acetic acid) for one hour and later, destained in 10 % acetic acid overnight. Gels were then scanned using an Epson Perfection V700 scanner. Gel image analyses were carried out using the GelAnalyzer 2010a software (gelanalyzer.com). The protein bands were classified into three groups according to He et al. (2013) and Wan et al. (2014). The first group corresponded to the HMW-GS, the second group to the  $\omega$ -gliadins and the third group combined a mixture of LMW-GS, and  $\alpha$ -/ $\gamma$ -gliadins.

#### 2.5. Protein extraction and SE-HPLC

For SE-HPLC (size-exclusion high performance liquid chromatography) analysis, 16.5 mg wholegrain flour were suspended in 1.5 mL of 5% SDS in sodium dihydrogen phosphate (pH 6.9) and then stirred for 5 minutes. Samples were then sonicated for 40 seconds, followed by centrifugation (30 min at 10000 g) to obtain the proteins in the supernatant. Separation was achieved in 30 min by loading 20  $\mu$ L of sample into an eluent (50% (v/v) acetonitrile and water, containing 1 mL L<sup>-1</sup> trifluoroacetic acid (TFA)) at a flow rate of 0.2 mL min<sup>-1</sup> using a Shimadzu Prominence HPLC and a Phenomenex BioSep<sup>TM</sup> SEC s4000 column. Proteins were detected by UV absorbance at 210 nm. The SE-HPLC chromatograms were divided into five sections of decreasing molecular size according to (He et al., 2013): large glutenin polymers (F1), small glutenin polymers (F2),  $\omega$ -gliadins (F3),  $\alpha$ -/ $\gamma$ -gliadins (F4) and non-gluten proteins (F5).

#### 2.6. Statistical analysis

The GenStat (17<sup>th</sup> edition, VSN International Ltd., Hemel Hempstead, UK) software was used for statistical analyses. Data were analysed by ANOVA, with the design structure of the experiment (randomised plots) being taken into account. Comparisons of relevant means

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were made using the least-significant difference at the 5 % level ( $p=0.05$ ). For the statistical analysis of the gel electrophoresis data, in addition to the field structure, the structure of the laboratory analysis (technical replicates) was included. For statistical analysis, the protein composition data from SDS-PAGE were converted to square root scale to assure variance homogeneity. Regression analysis was used to evaluate the relationships between grain protein concentration, gluten protein or the amount of gliadins and bread volume.

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## 3. Results

### 3.1. Grain yield and GPC

Three varieties were grown in a randomised field experiment at Rothamsted, UK at two fertilisation levels of nitrogen ( $100 \text{ kg N ha}^{-1}$  and  $200 \text{ kg N ha}^{-1}$ ). An additional dose of foliar N was applied at anthesis to one half of each plot. The three tested wheat varieties belong to different quality groups. Skyfall is a high yielding bread wheat with consistent quality in NABIM (National Association of British and Irish Millers) group 1. Soissons is an early maturing variety from NABIM group 2. Conqueror is a hard-milling feed wheat and belongs to NABIM group 4. Grain yield was enhanced by a higher N fertiliser level in both years except for Soissons in 2017 (Figure 1 A). On average, the higher N fertiliser level resulted in an additional grain yield of  $2.9$  and  $1.2 \text{ t ha}^{-1}$  in 2016 and 2017, respectively. An increased grain yield due to foliar N application at anthesis was only observed for Skyfall at N100 and for Soissons at N200. In 2016 at the  $100 \text{ kg N ha}^{-1}$  level, the grain yield of the feed wheat cultivar Conqueror was significantly ( $p < 0.05$ ) higher than that of the bread making cultivar Soissons, but there were no cultivar differences observed at the  $200 \text{ kg N ha}^{-1}$  level. In 2017, Soissons had a lower grain yield than Conqueror and Skyfall at both N fertilisation levels. GPC (calculated as grain N concentration  $\times 5.7$ ) was increased by a higher N fertiliser level as well as foliar N application at anthesis (Figure 1 B). Without foliar N application at anthesis, the average grain protein concentration was  $8.9 \%$ , which was raised to  $10.3 \%$  by foliar N application. There were significant ( $p < 0.05$ ) differences in GPC between cultivars. The lowest grain protein concentration was observed for Conqueror with  $8.7 \%$ , which was expected as Conqueror is a feed wheat. Contrary to the NABIM classification, GPC of Soissons ( $10.6 \%$ ) exceeded that of Skyfall ( $9.6 \%$ ). However, considering the average GPC, both varieties did not reach the standard specification for bread-making of at least  $12 \%$ . This minimum GPC

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for bread-making wheat was only achieved by Soissons in 2017 at the high N level (N200) combined with the foliar N application. GPCs for all varieties and N fertiliser levels were lower in 2016 than in 2017.

GPCs achieved at N200 + foliar N ranged from 9.8 % (Conqueror) to 11.9 % (Soissons) in year 2016 and from 11.2 % (Conqueror) to 13.5 % (Soissons) in year 2017. The minimum protein concentration of 12 %, required for bread-making in the U.K. (Godfrey et al., 2010; He et al., 2013) was only reached by the two bread-making wheat varieties tested in one of the two years.

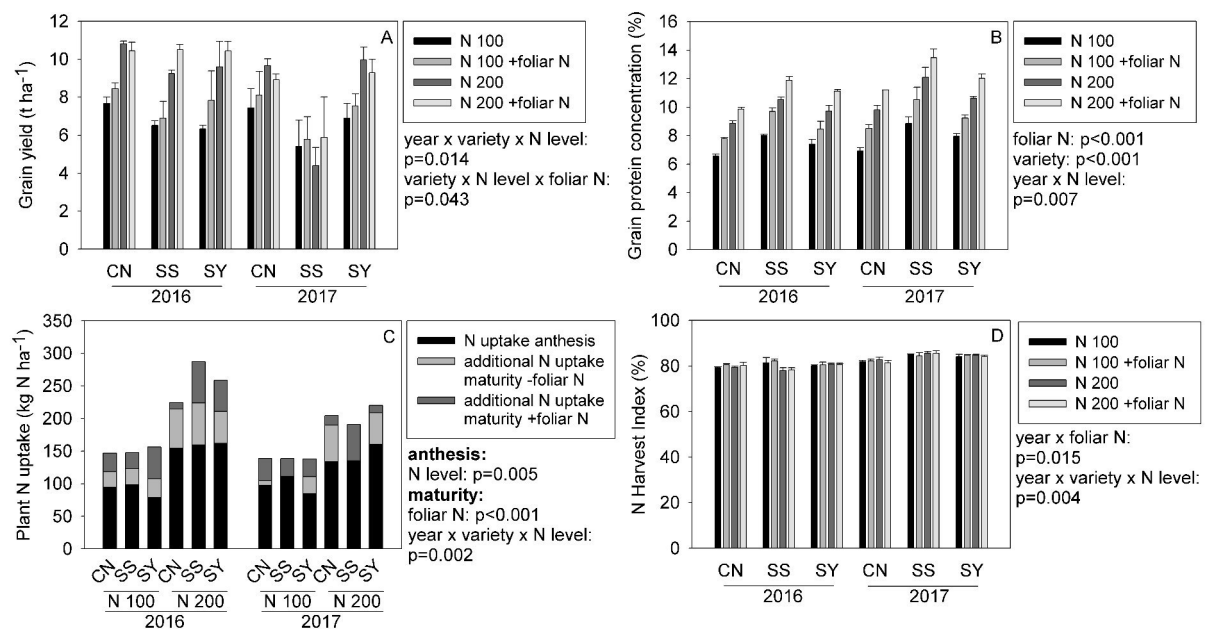


Figure 1: Grain yield (A), GPC (B), plant N uptake (C) and N harvest index (D) of the cultivars Conqueror (CN), Soissons (SS) and Skyfall (SY) at the two N fertilisation levels 100 kg N ha<sup>-1</sup> (N100) and 200 kg N ha<sup>-1</sup> (N200), with and without foliar N application at anthesis, in years 2016 and 2017. Error bars represent standard deviations of replicates.

#### 3.2. Plant N uptake and nitrogen harvest index

Plant N uptake was calculated from N concentration and biomass data at anthesis and maturity; the results are presented in Figure 1 C. At anthesis plant N uptake was equal for all

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three varieties with higher N uptake at the higher N fertiliser level. For N100, plants absorbed 94 kg N ha<sup>-1</sup> on average until anthesis and 151 kg N ha<sup>-1</sup> on average for N200. When no foliar N was applied, the average additional N uptake post anthesis was 16.7 and 42.5 kg ha<sup>-1</sup> for N100 and N200, respectively. When foliar N was applied, the additional N uptake post anthesis increased to 48.6 kg ha<sup>-1</sup> at N100 and to 76.3 kg ha<sup>-1</sup> at N200. An additional foliar N application at anthesis increased N uptake at maturity by 33 kg ha<sup>-1</sup>. This is consistent with the foliar-applied 40 kg ha<sup>-1</sup> N at anthesis. The higher N fertiliser regimen resulted in significantly ( $p < 0.05$ ) higher plant N uptake at maturity in all three cultivars.

The N-HI (nitrogen harvest index) was enhanced by a foliar N application at anthesis only in 2016. With an average of 80.1 %, the N-HI in 2016 was lower than in 2017 (83.9 %) as is shown in Figure 1 D. Statistical analysis showed a three-way interaction of the factors year, cultivar and N level on the N-HI. Part of this interaction is a decrease of the N-HI due to a higher N fertilisation level, but this effect only appeared in the cultivar Soissons and only in year 2016. In that year, Soissons had a higher N-HI than Skyfall and Conqueror at N100, but at N200 the N-HI for Soissons was the lowest. In 2017, the lowest N-HI was observed for Conqueror (82.1 %). There was no difference between Skyfall and Soissons at N100, but at N200 the N-HI of Soissons (85.6 %) was higher than that of Skyfall (84.5 %).

#### 3.3. Weather conditions during grain development

Weather conditions after anthesis in 2017 differed from those in 2016. Weather data for the variety Soissons and the varieties Skyfall and Conqueror are presented separately (Figure 2 and Figure 3) because in year 2016 Soissons' development was approximately one week earlier than the development of Skyfall and Conqueror. In a period from 15 to 23 DPA for Soissons and from 17 to 22 DPA for Skyfall and Conqueror, daily average temperatures were

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higher in 2017 than in 2016 (Figure 2 A). This led to higher accumulated degree days in 2017 from 17 DPA onwards for Skyfall and Conqueror, for Soissons the accumulated degree days in 2017 lay above those in 2016 for the whole period that was monitored (Figure 2 B). The accumulated rainfall and duration of sunshine during grain development are displayed in Figure 3. Both parameters show considerable differences between the two years of the experiment. In 2017, the total rainfall with 39 mm in a period from anthesis until 35 DPA was less than in 2016 (97 mm for Soissons and 118 mm for Skyfall and Conqueror). The duration of sunshine in the same period was 273 h for Soissons and 287 h for Skyfall and Conqueror in 2017, while in 2016 it was only 171 h (Soissons) and 220 h (Skyfall and Conqueror). For Soissons in 2017 there was 58 mm less rainfall and 102 more hours of sunshine during grain development compared to 2016. For Skyfall and Conqueror, there was 79 mm less rainfall and an additional 67 h of sunshine in 2017 in comparison with 2016.

### 3. Foliar N application effects on grain protein composition and baking quality

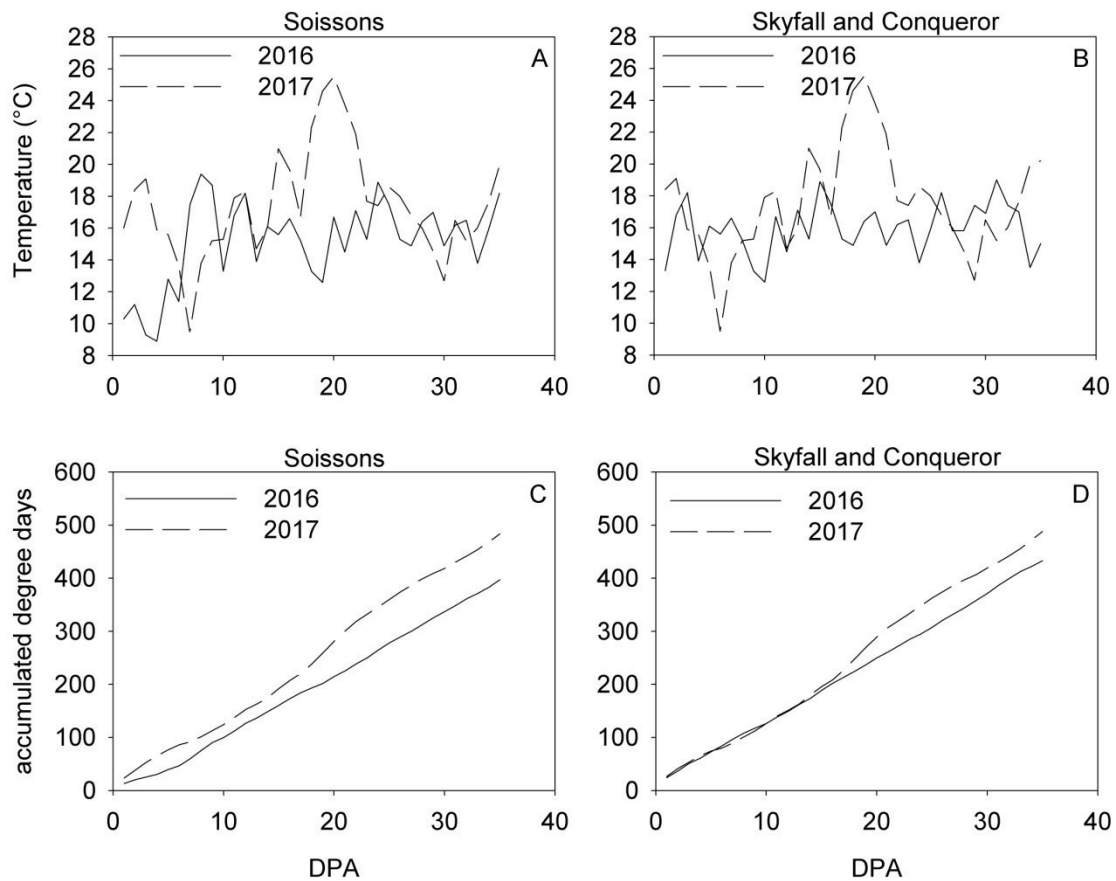


Figure 2: Daily average temperature (A and B) and accumulated degree days (C and D) during grain development (1-35 days post anthesis (DPA)) of the cultivars Conqueror, Soissons and Skyfall in the years 2016 and 2017.

### 3. Foliar N application effects on grain protein composition and baking quality

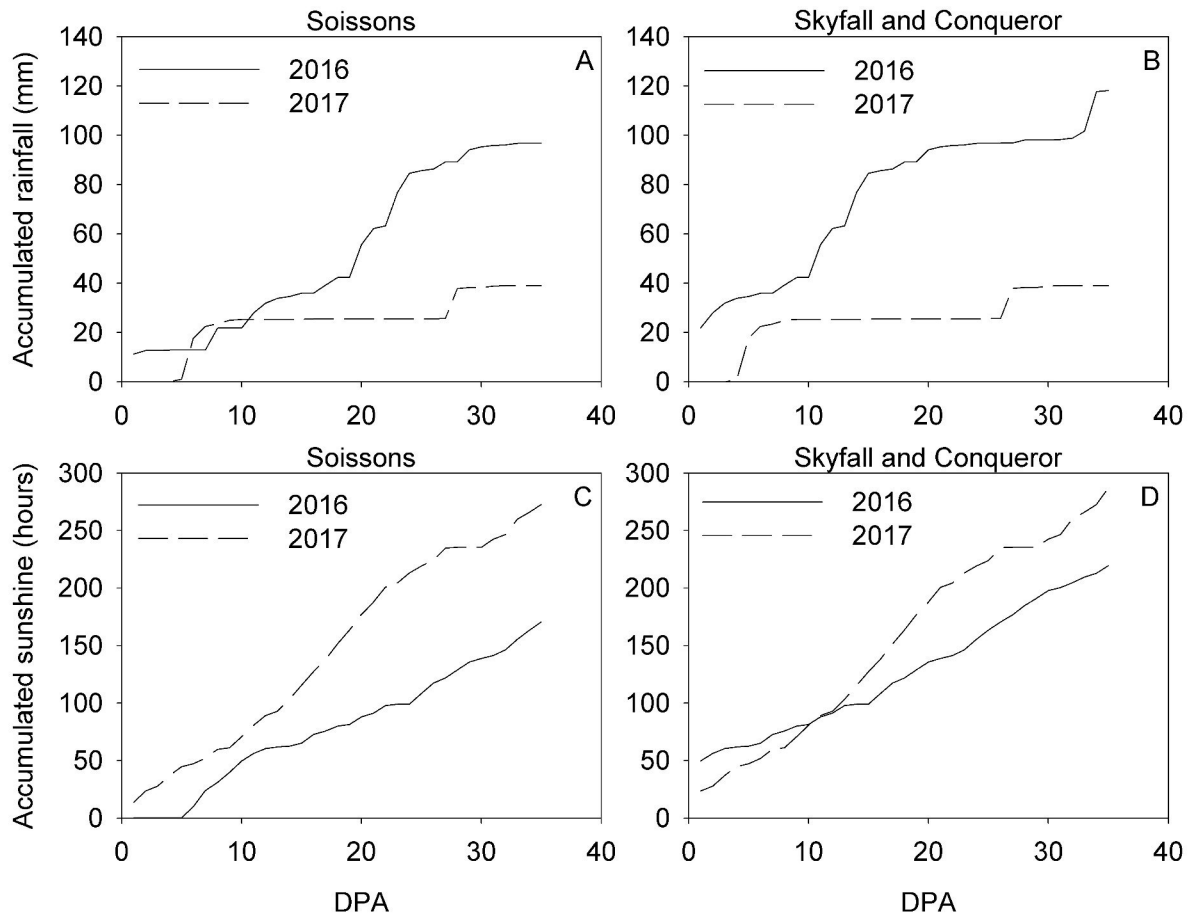


Figure 3: Accumulated rainfall (A and B) and sunshine (C and D) during grain development (1-35 days post anthesis (DPA)) for the cultivars Conqueror, Soissons and Skyfall, in the years 2016 and 2017.

#### 3.4. Thousand-grain-weight and Hagberg falling number

Grain size was measured as thousand-grain-weight (TGW). The TGW was increased by the foliar N application at anthesis at both N fertiliser levels (N100 and N200) as is shown in Figure 4 A. When more N was supplied (N200 vs. N100) TGW was decreased, though this effect was only present when foliar N was applied at anthesis. The highest TGW was realised with 45.7 g at N100 when foliar N at anthesis was applied. When comparing cultivars, an increased N fertiliser level reduced grain size for Conqueror and Soissons but not for Skyfall. At both N fertiliser levels, Soissons had the smallest grains with an average TGW of 37.72 g.



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TGW for Conqueror and Skyfall did not differ at N100, but at N200 Skyfall with a TGW of 46.37 g, had bigger grains than Conqueror (43.47 g).

Hagberg falling number (HFN) is a measure of the starch quality of flour produced from wheat grain. A falling number of less than 220 s is recognised as low, whereas a falling number greater than 300 s is high. The HFN was improved by 23.5 % due to the foliar N application at anthesis when N supply was low (N100) but at high N supply (N200) there was no effect of foliar N on HFN observed (Figure 4 B). A higher N fertiliser level only improved HFN when no foliar N was applied at anthesis. There was a strong gradient within the three tested varieties, with flour from Skyfall having the highest HFN (350.7 s), followed by flours from Soissons (318.1 s) and Conqueror (221.4 s).

### 3. Foliar N application effects on grain protein composition and baking quality

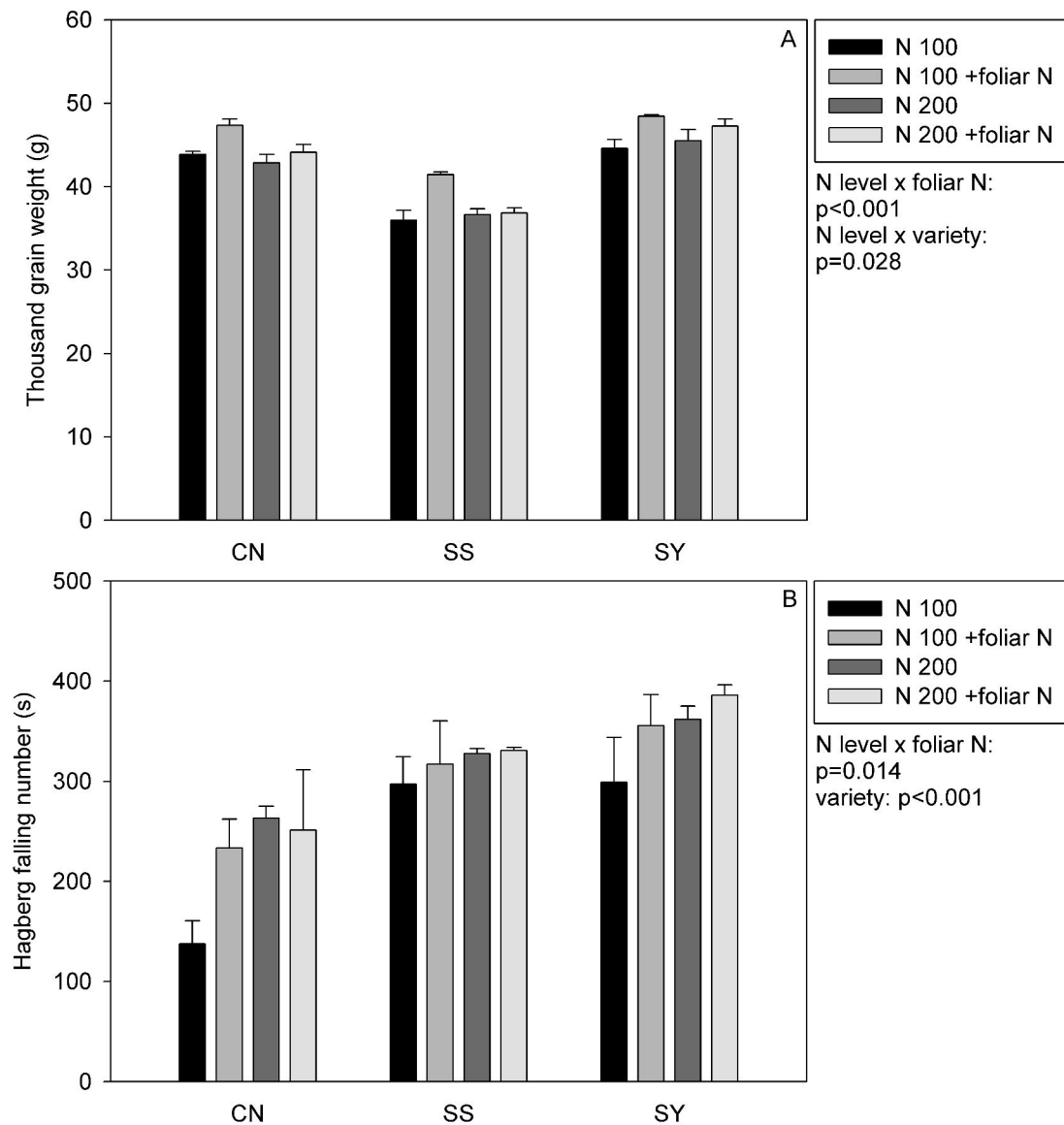


Figure 4: Thousand grain weight (A) and Hagberg falling number (B) of the cultivars Conqueror (CN), Soissons (SS) and Skyfall (SY), at the two N fertilisation levels  $100 \text{ kg N ha}^{-1}$  (N100) and  $200 \text{ kg N ha}^{-1}$  (N200), with and without foliar N application at anthesis. Error bars represent standard deviations of replicates.

#### 3.5. Bread volume

Bread volume was investigated by micro baking tests and results are summarised in Figure 5. The volume of breads baked from the flour of the variety Soissons, with 30.14 mL (mean over all N levels), was significantly higher than those of Skyfall and Conqueror (24.28 and

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23.36 mL, respectively). A foliar N application at anthesis improved bread volume only at the low N fertilisation level (N100). In general, bread volumes were higher at the high N fertilisation level (N200). The average bread volume at N100 with an additional dose of N applied via the leaves at anthesis (25.78 mL) was comparable with the average bread volume at N200 without a foliar N application at anthesis (27.41 mL).

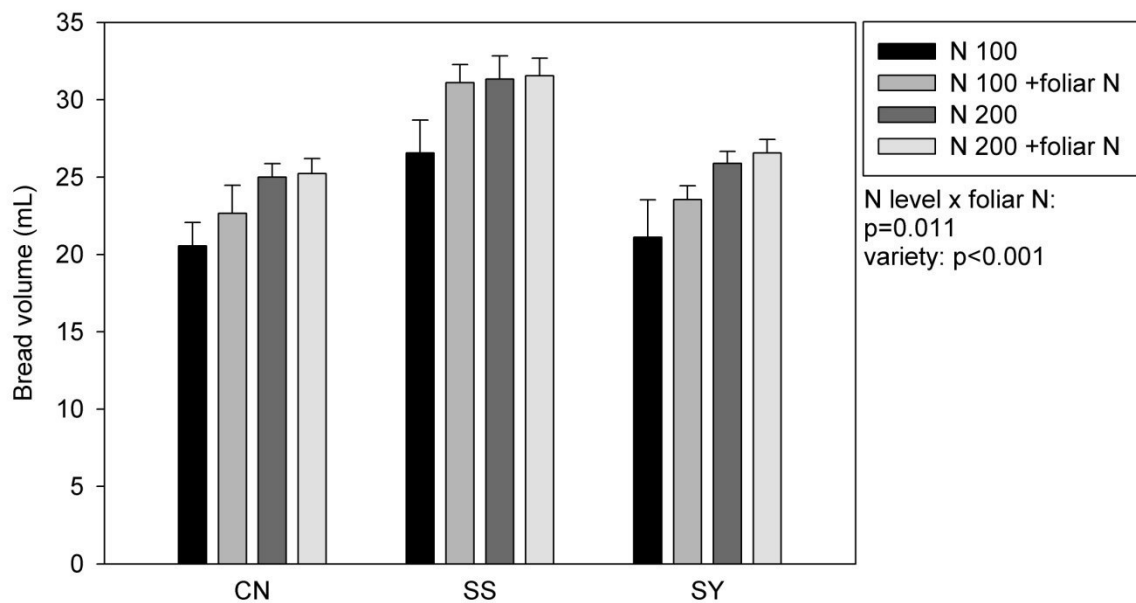


Figure 5: Bread volume in the cultivars Conqueror, Soissons and Skyfall as affected by the two N fertilisation levels 100 kg N ha<sup>-1</sup> (N100) and 200 kg N ha<sup>-1</sup> (N200), with and without foliar N application at anthesis. Error bars represent standard deviations of replicates.

#### 3.6. Protein composition, measured by SDS-PAGE

Extracted grain proteins were separated by size on the basis of their mobility using SDS-PAGE to analyse the gluten subunits. The protein bands were classified into three groups. The first group corresponded to the HMW-GS, the second group to the  $\omega$ -gliadins and the third group combined a mixture of LMW-GS, and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins. The SDS-PAGE results are shown in Figure 6 A.

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Considering the general increase in GPC with higher N fertiliser level and foliar N application at anthesis, the alterations in the composition of the storage protein fractions given as percentage from total extracted protein is of particular interest. The LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins were the major group of gluten proteins with an average of 90.7 %. The HMW-GS made up for 6.5 % whereas the  $\omega$ -gliadins accounted for 2.8 %. The percentage of  $\omega$ -gliadins was not changed by foliar N application at anthesis. The percentage of HMW-GS was increased by foliar N application while the percentage of LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins was decreased. The gluten protein composition was also influenced by variety. Soissons showed higher percentages of HMW-GS and  $\omega$ -gliadins than Skyfall and Conqueror. The percentage of LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins was lowest in Soissons (82.6 %) and highest in Conqueror (96.6 %).

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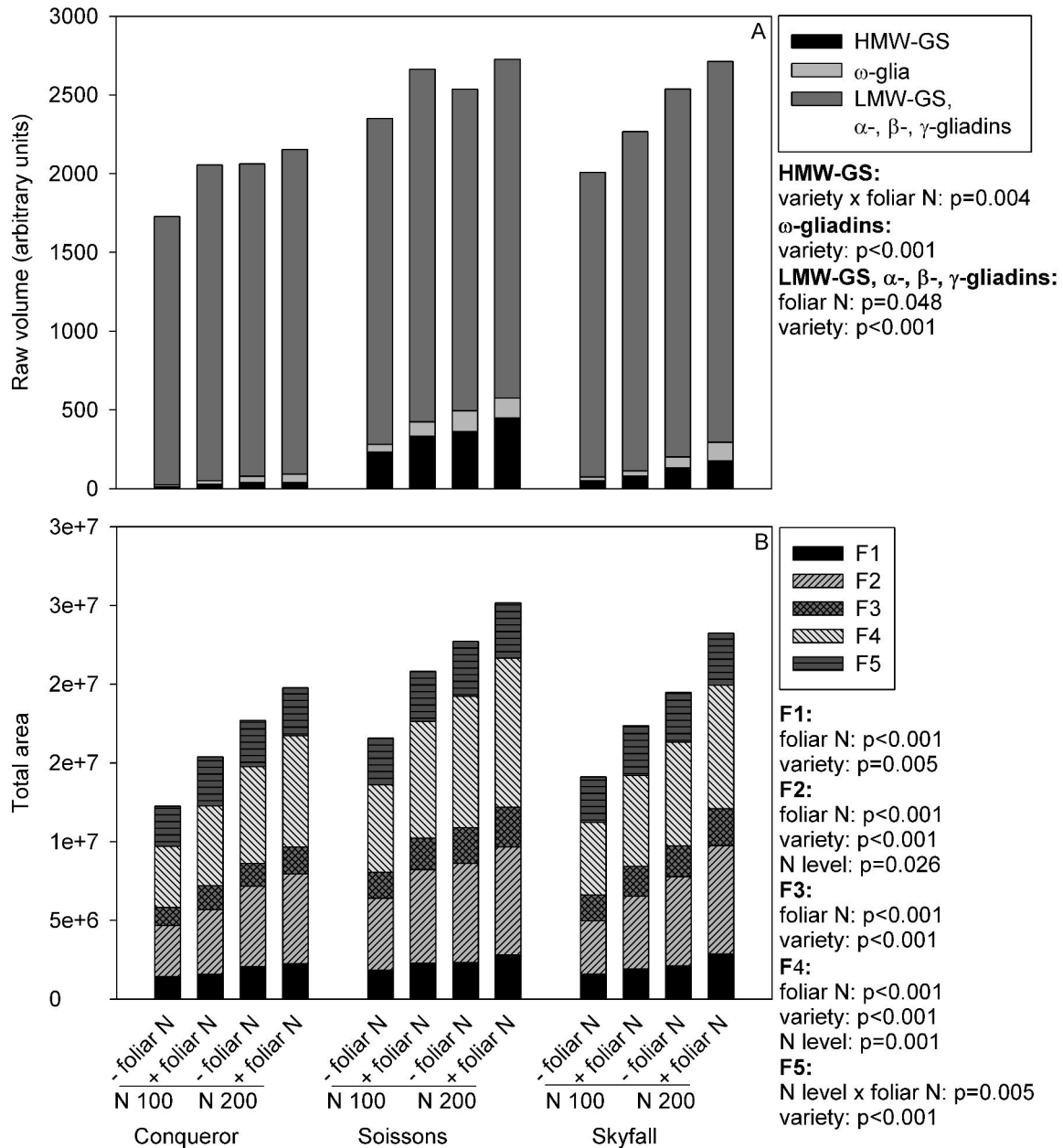


Figure 6: Proportions of protein fractions at maturity, measured by SDS-PAGE (A) and composition of protein polymers analysed by SE-HPLC (B) (displayed as area underneath the chromatogram), as affected by the two N fertilisation levels 100 kg N ha<sup>-1</sup> (N100) and 200 kg N ha<sup>-1</sup> (N200), with and without foliar N application at anthesis in the cultivars Conqueror, Soissons and Skyfall. **F1**: large glutenin polymers (containing HMW-GS), **F2**: small glutenin polymers (containing LMW-GS), **F3**: fraction enriched in  $\omega$ -gliadins, **F4**: fraction enriched in  $\alpha$ -/ $\gamma$ -gliadins and **F5**: containing non-gluten proteins.

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#### 3.7. Protein polymer composition, analysed by SE-HPLC

Storage proteins were analysed by size using SE-HPLC technique to analyse polymers. The results from this analysis are presented in Figure 6 B. The SE-HPLC chromatograms were divided into five sections, representing different storage protein fractions: large glutenin polymers (high molecular weight glutenin subunits (HMW-GS)) (F1), small glutenin polymers (low molecular weight glutenin subunits (LMW-GS)) (F2),  $\omega$ -gliadins (F3),  $\alpha$ -/ $\gamma$ -gliadins (F4) and non-gluten proteins (F5). (F1+F2+F3+F4+F5) represents the total protein amount, whereas (F1+F2+F3+F4) represents the amount of gluten protein.

While the total amounts of HMW-GS, LMW-GS,  $\omega$ -gliadins and  $\alpha$ -/ $\gamma$ -gliadins were increased by a foliar N application at anthesis at both, low and high N fertilisation level, the amount of the non-gluten proteins was increased solely at the low N fertiliser regimen (Figure 6 B). Only the LMW-GS and  $\alpha$ -/ $\gamma$ -gliadins were enhanced by a higher N fertilisation level. For all storage protein groups, there was a gradient between the three tested cultivars, with Conqueror showing the least amounts of each protein group, followed by Skyfall and then Soissons with the highest amounts. The HMW-GS are the only exception from this pattern, with Skyfall and Soissons showing similar amounts, both higher than Conqueror.

Ratios between certain storage protein fractions, such as HMW-GS/LMW-GS ratio, gliadin/glutenin ratio and gliadin/HMW-GS ratio  $((F3+F4)/F1)$ , are said to indicate for the baking quality (Godfrey et al., 2010; Millar, 2003). The ratio of HMW-GS to LMW-GS is given by  $F1/F2$ , the gliadin/glutenin ratio is calculated by  $(F3+F4)/(F1+F2)$  and the ratio of gliadin to HMW-GS is represented by  $(F3+F4)/F1$ . Both, HMW-GS/LMW-GS ratio and gliadin/HMW-GS ratio were changed due to the foliar N application at anthesis, but only at the low N fertilisation level (Figure 7). The HMW-GS/LMW-GS ratio was decreased, whereas the gliadin/HMW-GS ratio was increased by the foliar N application. At the low N fertiliser level

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(N100), the amount of gliadins was increased by 33 % as a result of foliar N application at anthesis, whereas the amount of HMW-GS was increased by 19 %. As the increase in gliadins was stronger than that of HMW-GS, the ratio of gliadins to HMW-GS was enhanced by foliar N application at anthesis under a low N fertiliser regimen (Figure 7 B). The gliadin/glutenin ratio remained unaffected.

### 3. Foliar N application effects on grain protein composition and baking quality

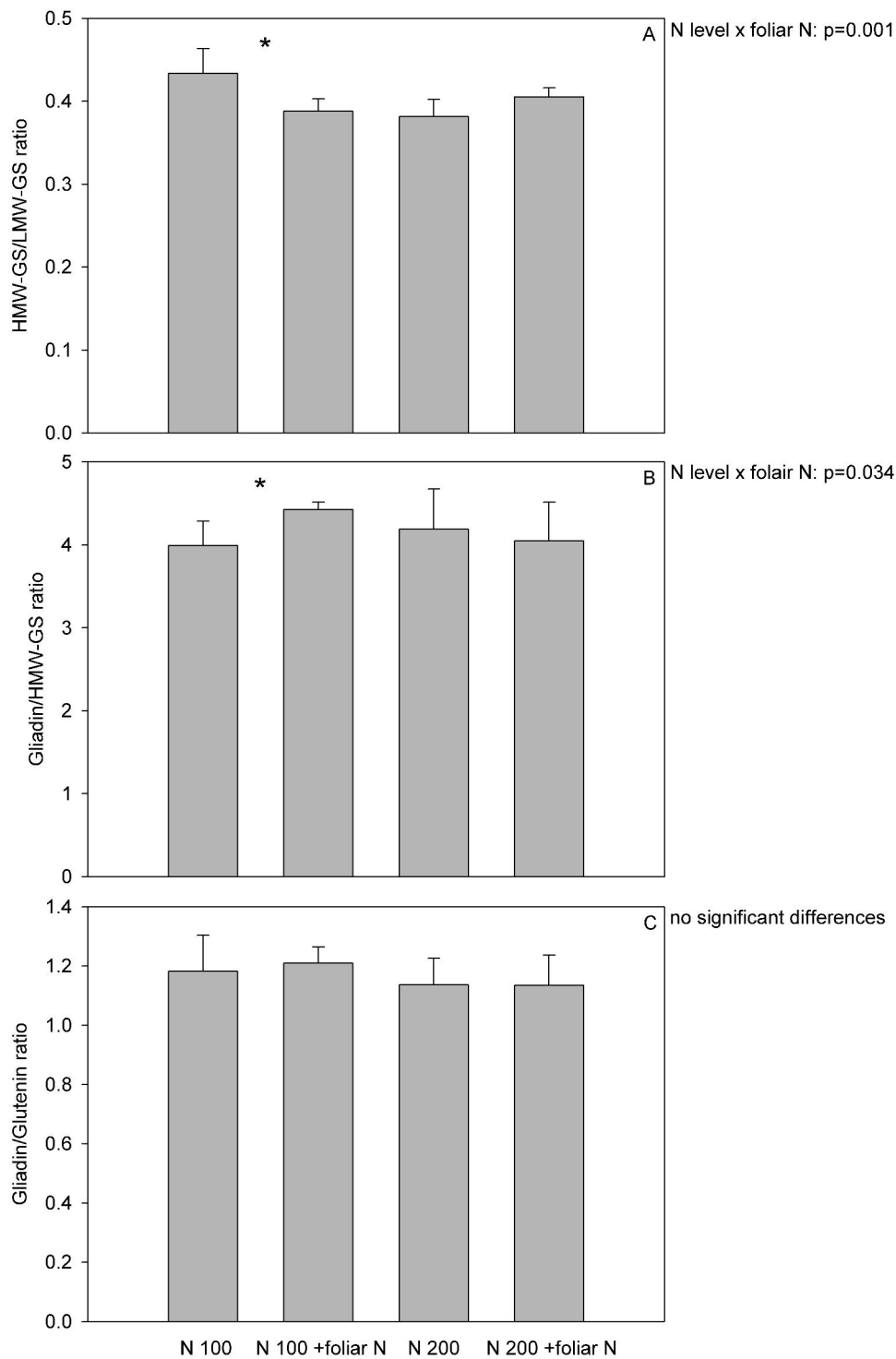


Figure 7: HMW-GS/LMW-GS ratio (A), gliadin/HMW-GS ratio (B) and gliadin/glutenin ratio (C) at the two N fertilisation levels 100 kg N ha<sup>-1</sup> (N100) and 200 kg N ha<sup>-1</sup> (N200), with and without foliar N application at anthesis. Error bars represent standard deviations of replicates. Asterisks mark significant effects of a foliar N application (p < 0.05)



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#### 3.8. Relationships between some protein-related parameters and bread volume

Regression analyses were carried out to evaluate the relationships of all gluten protein fractions and subunits measured by SE-HPLC, results obtained from farinograph measurements, and in addition GPC, with bread volume. The correlations of the most promising three parameters (GPC, total amount of gliadins and total amount of gluten protein) are presented in Figure 8. The best correlation was found between bread volume and the amount of gliadins in the flour with a correlation coefficient of 0.718. When regression analyses were performed for the varieties separately, the correlations were weakest for Soissons and strongest for Conqueror.

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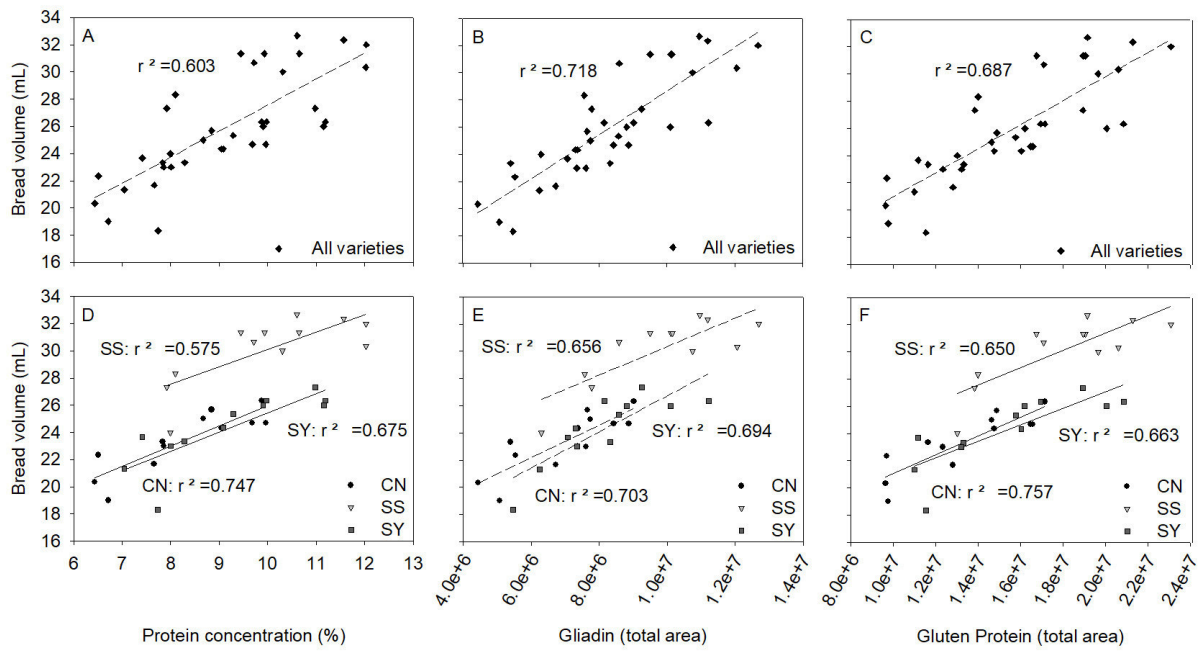


Figure 8: Correlations between bread volume and protein concentration (A and D), bread volume and the amount of gliadins measured by SE-HPLC (B and E), and bread volume and the amount of gluten protein measured by SE-HPLC (C and F) for all three varieties (A, B and C) and for each of the three varieties (Conqueror (CN), Soissons (SS) and Skyfall (SY)) (D, E and F).

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## 4. Discussion

#### 4.1. Plant N uptake effects on grain yield and GPC

As nitrogen is an essential component of grain proteins, plant N uptake is crucial for grain yield production as well as storage protein accumulation during grain development. When more N (N100 vs. N200) was supplied, plants assimilated more N until anthesis. This plant N uptake until anthesis results in both a higher grain yield and a greater GPC. The increased plant N uptake at maturity due to a foliar N application at anthesis influenced grain yield only for Skyfall at N100 and for Soissons at N200, but led to an increased GPC in all three tested varieties at both N fertiliser levels (Figure 1). Therefore, it can be assumed that the additional N, taken up after foliar N application at anthesis, was used primarily for grain protein synthesis. This finding goes along with results from Kichey et al. (2007) and Taulemesse et al. (2016), showing that major proportions of N absorbed during the post-anthesis period are translocated to the grain. Plant N uptake is an important measure, but under high N fertiliser regimes, grain N concentration usually reaches a maximum and then remains stable, whereas the N concentration in the straw fraction continues to rise (Barneix, 2007; Kong et al., 2016; Pask et al., 2012; Triboi and Triboi-Blondel, 2002). In this case, the N harvest index would be reduced. In this experiment, the higher N level only decreased N-HI in Soissons in one of the two years. For the cultivars Skyfall and Conqueror there was no such effect observed. These findings demonstrate that even a dose of 200 kg N ha<sup>-1</sup> can still be used effectively by wheat plants by translocating the absorbed N to the grains. The changes in GPC under different N fertiliser treatments also support this statement because GPC was enhanced by both a higher N fertiliser level and a foliar N application at anthesis.

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#### 4.2. Influences of the weather conditions during grain development on yield and protein concentration

Weather conditions strongly affect grain yield as well as grain quality of wheat (Johansson and Svensson, 1998; Schulz et al., 2015). The higher grain yield in 2016 can be explained by the lower temperature during grain development, which slows down the process of senescence and therefore extends the phase of grain filling. Johansson and Svensson (1998) observed a positive correlation between the hours of sunshine in May and June and the GPC as well as a negative correlation between the amount of rainfall in June and the GPC. These findings are supported by the results of our experiment which show less GPC in consequence of fewer hours of sunshine and more rainfall during grain development (Figure 1 and Figure 3).

#### 4.3. Thousand grain weight and Hagberg falling number as influenced by N fertiliser management

Grain yield is composed of parameters, such as seed rate, shoots per plant and grain size. TGW was increased by foliar N application at anthesis but there was no effect of foliar N application on grain yield (Figure 4 and Figure 1). It can be concluded that due to the foliar N application the wheat plants produced fewer but larger grains. Demotes-Mainard et al. (1999) found that the number of grains is usually determined before anthesis, which contradicts the conclusion indicated above. During sampling in the field, there were more sterile florets observed in plots which had received a foliar N application. This could be the reason for the impact of foliar N application on TGW even if the number of florets (potential grains) has been determined earlier. The foliar N application at anthesis only improved HFN at the low N fertilisation level (N100). This finding is consistent with the statement of

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Gooding and Davies (1992) that foliar application of urea can reduce  $\alpha$ -amylase activity and therefore improve HFN.

#### 4.4. Grain protein composition as influenced by N fertiliser management

The composition of grain protein determines the end-use quality of wheat flours and was analysed in samples from year 2016.

The results of SDS-PAGE show an increased amount of HMW-GS due to the foliar N application at anthesis in two of the three tested varieties (Skyfall and Soissons). The amounts of LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins were also positively affected by the foliar N application (Figure 6 A). The  $\omega$ -gliadins were the only group that remained unaffected by the application of foliar N. Daniel and Triboi (2000) and Hurkman et al. (2013) also report an increase of HMW-GS in response to N fertilisation but they found that  $\omega$ -gliadins increase as well and amounts of LMW-GS decrease.

Considering the results from the SE-HPLC analysis, it is noteworthy that even though the N fertiliser level increased GPC, the effect on the composition of grain proteins was rather small. The N fertiliser level only affected the amounts of the fractions F2 (LMW-GS) and F4 ( $\alpha$ -/ $\gamma$ -gliadins) but these changes caused a significant ( $p < 0.05$ ) influence of N fertiliser level on both total gliadins and total glutenins (Figure 6 B). This confirms the results from Xue et al., 2016b and Fuertes-Mendizábal et al., 2010, who also reported extended amounts of gliadins and glutenins due to a higher N fertilisation rate. The amounts of gliadins and glutenins were also influenced by an additional foliar N application at anthesis. Since both protein fractions were enlarged by 21 % as a result of a foliar N application, the ratio of gliadins to glutenins remained unchanged (Figure 7 C). Triboi et al. (2000) also observed

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equal increases of gliadins and glutenins in response to N supply and therefore, no changes in their ratio.

#### 4.5. Concentration and composition of grain protein and their connection to bread volume

Grain N concentration was enhanced by N level as well as foliar N application (Figure 1 B). As the bread volume only increased due to a foliar N application at a low N fertilisation rate (N100), it can be concluded that an increasing grain protein concentration does not necessarily lead to an improved baking quality. Baking performance rather depends on the composition of grain proteins. Millar (2003) states that bread-making varieties generally tend to have higher HMW-GS/LMW-GS and lower gliadin/HMW-GS values. However, the results of our experiment show the opposite effect. At a low N fertiliser level (N100), the foliar N application at anthesis decreased the HMW-GS/LMW-GS ratio and increased the gliadin/HMW-GS ratio. These alterations led to an improved bread volume. Interestingly, the foliar N effect on the ratios of HMW-GS/LMW-GS and gliadin/HMW-GS only occurred when N fertiliser supply was low (N100). No such effect was observed at the high N fertilisation level. The changes in bread volume follow the same pattern: bread volume was increased by foliar N application at low N fertiliser level (N100) but not when N supply was high (N200). The increased GPC due to foliar N application at the high N fertilisation level did not lead to further improvements in bread volume.

Surprisingly, the baking performance of the bread-making variety Skyfall was comparable to that of the feed wheat variety Conqueror. A possible explanation for the poor bread volume of Skyfall could lie in the flour type used for the micro-scale baking test. As wholemeal flour was used for the baking tests and Skyfall is a variety designed for the British market, where there is mainly bread consumed which is produced from white flour, this might have

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particularly affected the results of Skyfall. It can be speculated that Skyfall is unsuitable for being processed in the form of wholemeal flour but might show a good baking performance when used in form of white flour.

The parameter which could explain variance in bread volume in our experiment best was the total amount of gliadins measured by SE-HPLC ( $r^2=0.718$ ). Grain protein concentration only accounted for 60 % of the variance in bread volume when all three varieties were considered. When regression analysis was performed separately for the varieties, the correlation was weakest for Soissons ( $r^2=0.575$ ) which had the highest GPC and strongest for Conqueror ( $r^2=0.747$ ) which had the lowest GPC. This finding confirms the weaker relationship between GPC and bread volume in high protein varieties discovered by Gabriel et al. (2017). This demonstrates that the evaluation of bread making quality of wheat flour should not depend on GPC alone but needs consideration of storage protein composition as well.

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## 5. Conclusion

In this study, an increasing GPC did not necessarily generate an improved baking quality, as the increased GPC due to foliar N application at the high N fertilisation level did not lead to further improvements in bread volume. Baking performance rather depends on the composition of grain proteins. At a low N fertiliser level (N100), the foliar N application at anthesis decreased the HMW-GS/LMW-GS ratio and increased the gliadin/HMW-GS ratio, which led to an improved bread volume. No such effects were observed at high N fertilisation level (N200). In this study, the parameter which could explain variance in bread volume best was the total amount of gliadins.

These results imply that a late foliar application of nitrogen can be used to effectively improve baking quality in case the total N uptake was low due to unfavourable weather conditions during growing season until anthesis. The findings also show that this effect cannot be observed by measuring GPC alone but also needs a combined inquiry of storage protein composition as well as bread volume. For the future, a quickly detectable parameter for precise bread making quality predictions still needs to be developed. One possibility is that this parameter should be based on the total amount of gliadins.



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4. Effects of a foliar N application on gene expression of protein fractions and protein body distribution

## Chapter 4

Foliar N application at anthesis stimulates gene expression of grain protein fractions and alters protein body distribution in winter wheat (*triticum aestivum* L.)

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to be submitted

# Foliar N application at anthesis stimulates gene expression of grain protein fractions and alters protein body distribution in winter wheat (*triticum aestivum* L.)

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#### 4. Effects of a foliar N application on gene expression of protein fractions and protein body distribution

##### **Abstract**

The major components of wheat storage proteins are gliadins and glutenins, and as they contribute differently to baking quality, a balanced mixture of these components is essential. Applying foliar nitrogen (N) at anthesis is a common practice to improve protein concentration and composition. The aim of this study was to investigate the effects of a foliar N application at anthesis on storage protein gene expression during grain development and on the distribution of protein concentration and protein body size within the grain. In this experiment an additional N application at anthesis stimulated expression of genes of the majority of storage proteins when the N supply was low. Furthermore, it led to higher protein concentrations in the sub-aleurone layers, while in the centre of the lobes, the protein concentrations were decreased. These changes will affect the protein recovery in white flours, as proportionally more protein might be lost during milling processes.

**Keywords:** wheat, gluten protein, gene expression, grain protein distribution, protein body size distribution

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### 1. Introduction

The ability of flours from wheat grain to form an elastic network is unique. The composition of proteins in wheat grain is of particular importance as it determines dough rheological properties and therefore baking quality. Gliadins and glutenins are major components of wheat storage proteins and influence bread-making quality differently. The polymeric glutenins consist of high and low molecular weight glutenin subunits (HMW-GS, LMW-GS, respectively), which are cross-linked by disulphide bonds.<sup>1</sup> They contribute to dough elasticity and strength by forming a continuous network. The monomeric gliadins work as plasticisers of the glutenin network and therefore reduce elasticity.<sup>1-3</sup> Hence, to achieve a good baking quality with viscoelastic dough properties, a balanced mixture of gliadins and glutenins is essential. As storage proteins accumulate in wheat grains during the grain filling and maturation phase after anthesis, it is important to gain a comprehensive knowledge about molecular processes involved, and interacting factors. It is known that the transcripts of storage proteins are most abundant in developing wheat grain, and that gene expression of many related genes undergoes massive changes during the time between anthesis and maturity.<sup>4</sup> There have also been investigations on gene expression of specific storage protein families, over time courses during grain development.<sup>5-8</sup> Wan et al. (<sup>9</sup>) studied the impact of N fertilizer level on the expression of  $\omega$ -gliadin transcripts, but until now, there is no knowledge of the influence of a foliar application of nitrogen at anthesis on gene expression of storage proteins.

There are two pathways of protein transport with proteins being either aggregated within the lumen of the rough endoplasmic reticulum (ER), or being transported via the Golgi apparatus into the vacuoles.<sup>10,11</sup> The two pathways appear to result in two types of protein bodies. Those protein bodies originated in the ER are enriched in HMW-GS, whereas the



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other protein bodies have high contents of gliadins – especially in young grains<sup>10</sup>. Protein bodies of different sizes also accumulate different gluten proteins. While gliadins and LMW-GS are more abundant in smaller protein bodies close to the aleurone layer, HMW-GS are enriched in bigger protein bodies, which appear in the sub-aleurone layer as well as in the centre of the endosperm.<sup>12</sup> The influences of post-anthesis temperature and N fertilization level on the protein body distribution within the wheat grain have been demonstrated recently.<sup>13</sup>

A foliar N application at anthesis is a common method to improve the protein concentration and composition. It holds advantages compared to soil application as plants can take up N even in dry weather conditions when N is unavailable in the soil. Furthermore, N losses through nitrate leaching into the ground water and denitrification can be reduced.<sup>14</sup> In this context, the aim of this study is to investigate the effects of a foliar N application at anthesis on the distribution of protein bodies within the grain and to better understand the underlying mechanisms of wheat quality.

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## 2. Experimental

### 2.1. Plant cultivation and sampling

Three wheat cultivars were grown in a randomised block design field experiment at Rothamsted at two different N fertilizer levels, 100 kg N ha<sup>-1</sup> (N100) and 200 kg N ha<sup>-1</sup> (N200) in the years 2016 and 2017, as part of the UK Defra WGIN project <sup>15</sup>. An additional dose of 40 kg N ha<sup>-1</sup> as foliar spray application (Folex N (urea), OMEX, KingsLynn, UK)) was applied at anthesis (growth stage 65), to one half of each 3 x 2.5 m plot. Thus, there were four different N treatments tested: N100, N100 +foliar N, N200, N200 +foliar N. Ears were tagged at anthesis (growth stage 65) and caryopses were harvested at five time points after anthesis (10, 14, 21, 28 and 35 days post anthesis (DPA)) for gene expression measurement. Harvested caryopses were immediately frozen in liquid N and stored at -80 °C. For light-microscopy analysis, developing grains were collected at 21 DPA from the first floret in spikelets from the centre of the ear. This way a comparable developmental status of collected caryopses was ensured.

### 2.2. Grain N concentration

Caryopses were ground in a freezer mill (Freezer Mill 6870, Spex SamplePrep, Stanmore, UK) and aliquots were freeze dried for at least 36 hours. The nitrogen concentration of the samples was measured by the Dumas method using a LECO CN628 Combustion Analyser (LECO Corporation, St Joseph, Michigan, USA).

### 2.3. Protein extraction and SDS-PAGE

Grain samples collected at five time-points after anthesis (10, 14, 21, 28, 35 DPA) were ground using a freezer mill (Freezer Mill 6870, Spex Sample Prep, Stanmore, UK). Aliquots of each sample were freeze-dried for at least 36 hours. Total gluten protein fraction was

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extracted from 10 mg flour using 50% (v/v) propan-1-ol + 2% dithiothreitol (DTT) at 50 °C, the extraction being repeated and the supernatants combined. After freeze-drying, the protein was dissolved in lysis buffer (8 M urea, 2 M thiourea, 4 % CHAPS, 30 mM DTT, 20 mM Tris base). The 2-D Quant Kit (GE Healthcare, Germany) was used to quantify the extracted protein. The sample amount containing 10 µg protein was combined with loading buffer (50 mM Tris-HCl, pH 6.8, 2 % SDS, 10 % glycerol, 0.1 % bromophenol blue, 200 mM DTT) and loaded on 7 cm precast gels (4-15 % Mini-PROTEAN TGX Precast Protein Gels, Biorad, Watford, UK) for 1D SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Precision Plus Protein™ Standard (Biorad, Watford, UK) was applied to a single well in each gel. Running conditions were 40 V for 30 minutes, followed by 80 V for 120 minutes. After completion of the run, gels were fixed (40 % ethanol, 50 % H<sub>2</sub>O and 10 % acetic acid) for one hour. Afterwards, gels were stained (1 Coomassie tablet (GE Healthcare, Germany) in 1.6 L 10 % acetic acid) for at least one hour and then destained in 10 % acetic acid overnight. Gels were scanned using an Epson Perfection V700 scanner. Gel images were analyzed using the GelAnalyzer 2010a software ([gelanalyzer.com](http://gelanalyzer.com)).

#### 2.4.RNA extraction and gene expression measurement

Total RNA was isolated from frozen, ground wheat caryopses, harvested at 10, 14, 21, 28, and 35 DPA, using a modified protocol from Verwoerd et al. (<sup>16</sup>) involving an additional phenol-chloroform-isoamyl alcohol extraction and DNase treatment (Promega, Madison, USA). Integrity of the RNA and absence of genomic DNA was confirmed on a 1.5 % (w/v) agarose gel. After final ethanol precipitation, total RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water. The concentration of extracted RNA was measured with a spectrometer (Nanodrop 2000c, Thermo Scientific). 2 µg of total RNA was used for poly-T primed reverse transcription with Superscript™III reverse transcriptase

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(Invitrogen, Thermo Fisher Scientific). 1 µl of 1:2 cDNA diluted and 250 nM primer was used for real-time PCR expression analysis in a 25 µL reaction with SYBR® Green JumpStart™ Taq ReadyMix (Sigma-Aldrich, UK). Real-time PCR was performed on an ABI 7500 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific). Relative expression of genes of the following six storage protein families was measured: high molecular weight glutenin subunits (HMW-GS), low molecular weight glutenin subunits (LMW-GS), α/β-gliadins, γ-gliadins 1 and 2, ω-gliadins. Gene expression was measured over a time course starting at 10 DPA and ending at 35 DPA. The stable 18S and 28S ribosomal RNA genes were chosen to determine the normalised quantification of expression. Sequences of all used primers are summarised in Table 1. Mean primer efficiencies were estimated using the linear phase of all individual reaction amplification curves<sup>17</sup> calculated using the LinRegPCR package<sup>18</sup>. The normalised relative quantity (NRQ) of expression was calculated in relation to the CT values and the primer efficiency (E) of both the target gene (X) and the mean of the two normalisation reference genes (N) as Normalised Relative Expression (NRE) based on<sup>19</sup>:

$$NRE = \frac{E_X^{(-CT_X)}}{\sqrt{(E_N^{(-CT_N)})}}$$

Equation 1: Calculation of NRE (normalised relative expression)

#### 2.5. Light-microscopy

Cross sections of grains collected at 21 DPA were fixed with 4 % paraformaldehyde and 2.5 % glutaraldehyde. After stepwise dehydration in an ethanol series the grain sections were embedded in LR White Resin according to Tosi et al. (12). After polymerisation, resin-embedded grains were sectioned at 1 µm thickness using an Ultra Microtome (Ultracut, C. Reichert AG, Wien, Austria) and a diamond knife (HISTO, 6 mm, TAAB laboratories

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equipment limited, England). Sections were floated in water, collected on slides and dried on a hot plate at 80 °C. Sections were stained for protein bodies with 1 % Naphthol Blue Black in 7 % (v/v) acetic acid for 45 seconds. Slides were overlaid with a coverslip using DPX mounting medium (containing xylene and dibutyl phthalate). Grain sections were examined with a Zeiss Axiophot (Zeiss, Oberkochen, Germany) at 20x magnification. Digital images were produced using the MetaMorph Microscopy Automation and Image Analysis Software version 7.8.0.0 (Molecular Devices, Sunnyvale, CA, USA). To determine protein body distribution and protein body size distribution, images of grain cross sections were loaded into the custom toolbox in ArcMap™, part of the ArcGIS™ 10.4 software package (ESRI®, Redlands, CA, USA) (available as an ArcGIS™ python toolbox, doi: 10.5281/zenodo.1066914). An outline was manually drawn around the endosperm just beneath the aleurone layer. Five concentric zones were added to the wheat endosperm image, protein areas and non-protein areas were classified before running two analyses simultaneously: protein body distribution analysis and protein body size distribution analysis. The procedure of classifying protein and non-protein areas and running the analyses was repeated three times for each image. This method is described in detail by Savill et al. (<sup>13</sup>). Figure 1 gives an overview of the protein gradient analysis procedure.

#### 2.6. Statistical Analysis and data processing

The GenStat® statistical software (17<sup>th</sup> edition, VSN International Ltd., Hemel Hempstead, UK) was used for statistical analyses. Data were analysed by ANOVA, the design structure of the experiment (randomised plots) being taken into account. Gene expression data were subjected to a log<sup>2</sup> transformation and microscopy data to a square root transformation to ensure a Normal distribution and variance homogeneity. Linear mixed models were fit to both the distribution of protein concentration over the calculated zones and to the protein

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body size distribution data using the method of restricted maximum likelihood. Data from each image analysis technique were analysed separately.

For the protein body distribution data, a conversion was applied after the analysis. The protein concentration determined through microscopy image analysis was converted to the actual protein concentration assessed by nitrogen content measurement of the grain as described by Savill et al. (<sup>13</sup>) using the following equation.

$$\text{Conversion factor} = \frac{\text{Protein concentration from N measurement}}{100 * \frac{\text{Total protein area from microscopy image analysis}}{\text{Total grain area from microscopy image analysis}}}$$

The zone applied to each protein concentration measurement was converted from a factor to a variable to account for different grain sizes in different treatments. Therefore the distance between the outline and the midpoint of each zone was calculated and translated from pixels to micrometers ( $\mu\text{m}$ ).

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### 3. Results

#### 3.1. Grain N concentration during grain development

Grain N concentration was measured at five time points after anthesis and at maturity. Statistical analysis showed two three-way interactions between experimental factors. The first interaction is between N fertilizer level, foliar N application and time point ( $p=0.036$ ), whereas the second interaction is between N fertilizer level, variety and time point ( $p=0.008$ ). At all time points, and at both N fertilizer levels (N100 and N200), a foliar N application at anthesis led to increased grain N concentrations (Figure 2). Under the low N fertilizer regimen (N100), grain N concentration decreased during maturation until 28 DPA for Conqueror and Skyfall, and remained on that level until harvest. For Soissons, grain N concentration decreased until 35 DPA, but slightly increased again until harvest. When N supply was high (N200), all varieties showed a decreasing N concentration in the grain during grain development with a minimum being reached at 28 DPA. In the time from 35 DPA to harvest (65 DPA), grain N concentration increased again up to a level comparable to the one around 21 DPA. There was a clear difference observed between the varieties at most time points. The grain N concentration of Conqueror was the lowest, whereas Soissons had the highest grain N concentration.

#### 3.2. Relative gene expression of storage proteins during grain development

Relative gene expression of six storage protein gene families was measured by real-time PCR for caryopses harvested at 10, 14, 21, 28 and 35 DPA. Gene expression was determined for caryopses from three wheat cultivars growing under low ( $100 \text{ kg N ha}^{-1}$ ) and high ( $200 \text{ kg N ha}^{-1}$ ) N fertilizer regimes. The gene expression of all examined storage protein families was up regulated shortly after 10 DPA, reaching a maximum around 21 or 28 DPA. At the low N fertilizer level (N100), gene expression of the gene families of HMW-GS,  $\alpha$ -/ $\beta$ -gliadins,  $\gamma$ -

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gliadins 1 and 2 and  $\omega$ -gliadins was enhanced by the additional foliar N application at anthesis (Figures 3 and 5-8). Only the gene expression of LMW-GS did not show any significant response to the foliar N application at N100 (Figure 4 A-C). When N supply was high (N200), no effect of the foliar N application at anthesis on the gene expression level of HMW-GS,  $\alpha$ -/ $\beta$ -gliadins and  $\gamma$ -gliadins 1 and 2 was observed. Gene expression of LMW-GS and  $\omega$ -gliadins decreased due to the foliar N application at anthesis but the latter only at 10 DPA. Statistical analysis revealed an interaction between variety and time point for all examined gene families at both N fertilizer levels. The only exception was the LMW-GS gene family, which showed clear differences between cultivars over all tested time points at N200. Gene expression of the LMW-GS at N200 was highest for Conqueror, whereas gene expressions for Skyfall and Soissons did not differ (Figure 4 D-F).

#### 3.3. Grain protein composition during grain development

Extracted protein of wheat caryopses collected at five time points post-anthesis was separated by size using SDS-PAGE, which resulted in three fractions of proteins: HMW-GS,  $\omega$ -gliadins and LMW-GS with  $\alpha$ -,  $\beta$ -,  $\gamma$ -gliadins. Statistical analyses showed several interactions between factors for all three protein groups. For HMW-GS, the most complex interaction occurred between variety, N fertilization level and foliar N application. The percentage of HMW-GS of total extracted protein (gluten protein) was increased by a foliar N application at anthesis, with the exception of Skyfall at the high N fertilization level (N200) (Figure 9 A-C). In general, HMW-GS percentage was highest in Soissons and lowest in Conqueror. The factors N fertilization level and variety showed interactions with the time point of sampling post-anthesis. At 10 DPA, the percentage of HMW-GS was higher when the N level was low (N100). At 14 DPA, the HMW-GS percentage was the same for both N levels, while from 21 DPA on the higher N level led to increased percentages of HMW-GS. For the percentage of



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$\omega$ -gliadins as well as LMW-GS with  $\alpha$ -,  $\beta$ -,  $\gamma$ -gliadins, there were three two-way interactions observed: foliar N x time point, N level x time point and variety x time point. While foliar N application increased the percentage of  $\omega$ -gliadins of total extracted protein at 14, 28 and 35 DPA, a higher N fertilization level only increased the percentage of  $\omega$ -gliadins at 35 DPA and final harvest. Except for 10 DPA and final harvest, the percentage of the group of LMW-GS and  $\alpha$ -,  $\beta$ -,  $\gamma$ -gliadins was decreased by a foliar N application. A higher N fertilization level (N200) also led to declined percentages of this protein group from 21 DPA onwards. From 14 to 35 DPA, the percentage of LMW-GS,  $\alpha$ -,  $\beta$ -,  $\gamma$ -gliadins was highest for Conqueror and lowest for Soissons. At final harvest, there was no statistical difference between Conqueror and Skyfall but Soissons still showed the least percentage of LMW-GS,  $\alpha$ -,  $\beta$ -,  $\gamma$ -gliadins.

#### 3.4. Protein body distribution within the grain

The protein body distribution within grain cross sections was determined using light-microscopy and image analysis in developing wheat grains (21 DPA). Statistical analysis revealed a four-way interaction of the factors: N fertilizer level, variety, foliar N application and distance from the aleurone layer ( $p=0.003$ ). The effects of an additional foliar N application at anthesis on the distribution of protein bodies are shown in Figure 10. A foliar N application at anthesis led to steeper gradients of protein concentrations within the grain cross sections. The protein concentration in the outer regions of the starchy endosperm (near the aleurone layer) was increased by a foliar N application in all three varieties and in both N fertilization levels, whereas in the centre of the lobes the protein concentration was decreased, except for Skyfall at N100, where the effect was not statistically significant. When comparing cultivars, Soissons had the steepest slope with protein concentrations ranging from 13.7 % in the sub-aleurone area to 4.6 % in the centre of the lobes, followed by

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Conqueror with a range from 10.6 % to 5.5 %. The slope of protein concentrations in grain cross sections from Skyfall was the shallowest with a range from 10.6 % to 6.3 %.

##### 3.5. Protein body size distribution within the grain

Spatial analysis was used to investigate the protein body size distribution within cross sections of developing wheat grains (21 DPA). This provides information about the average area of detected proteins at different locations across the starchy endosperm. Statistical analysis revealed a four-way interaction of the factors: N fertilizer level, variety, foliar N application and (distance from the aleurone layer)<sup>2</sup>. Nonlinear predictions of the protein body size distribution are shown in Figure 11 A-C. When comparing varieties, the protein bodies of Soissons, with an average size of  $0.23 \mu\text{m}^2$ , were smaller than those of Conqueror and Skyfall ( $0.31 \mu\text{m}^2$  and  $0.30 \mu\text{m}^2$ , respectively). For Conqueror, an additional foliar N application at anthesis combined with low N fertilizer input (N100) led to bigger protein bodies in the sub-aleurone zone and smaller protein bodies in the remaining parts of the endosperm. When N supply was high (N200), a foliar N application increased protein body size in the sub-aleurone zone as well as in the centre of the lobes. Between those grain areas, protein body size was decreased (Figure 11 A). For Soissons, the foliar N effect on protein body size distribution at the low N fertilization level (N100) was comparable to the effect in Conqueror at N100. When N supply was high (N200), the size of most protein bodies in grains of Soissons was decreased but the size of protein bodies near the centre of the lobes did not change (Figure 11 B). For Skyfall, a foliar N application at anthesis increased protein body size across the whole endosperm when the N fertilizer level was low (N100), whereas at the high N fertilizer level (N200) protein body size was decreased by a foliar N application (Figure 11 C). Figure 11 D-F shows the percentage of protein bodies, categorised by size into one of seven groups from the total protein body quantity. An additional foliar N

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application at anthesis increased the percentage of smallest protein bodies ( $0.05-0.18 \mu\text{m}^2$ ) and decreased the percentage of protein bodies with a size from  $0.73$  to  $2.93 \mu\text{m}^2$  (group of third smallest proteins) in Conqueror and Skyfall but not in Soissons. The percentage of the biggest protein bodies ( $187.32-421.46 \mu\text{m}^2$ ) was increased by a foliar N application at anthesis for Soissons and Skyfall when N supply was low (N100) and for Conqueror when N supply was high (N200). In general, the bigger protein bodies were located either in the sub-aleurone zone or in the mid part of the grain (half way between aleurone layer and the centre of the lobes), while the smallest protein bodies were located in the centre of the lobes. The abundance of smaller protein bodies was much higher than that of bigger protein bodies and this remained unaffected by variety, N level and foliar N treatment.

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### 4. Discussion

The three varieties tested are classified into three different quality groups. Skyfall is a high yielding bread wheat with consistent quality in NABIM (National Association of British and Irish Millers) group 1, while Soissons is characterised as an early maturing variety from NABIM group 2. Both varieties are designed for bread making. Conqueror on the other hand is a hard-milling feed wheat and belongs to NABIM group 4. The lowest grain N concentration found for Conqueror is in accordance with the classification as a feed wheat variety, which usually has less N and therefore protein in the grain. During grain development, the grain N concentration decreased, which can be explained by a dilution effect. Other grain components, such as starch and water, accumulate during grain development and while the total amount of N remains stable or even increases, the concentration reduces. During final grain maturation, the opposite effect occurs. Due to desiccation, the water content in the grain reduces<sup>20</sup> and causes the grain N concentration to rise (Figure 2).

The results of the gene expression analysis show that generally, the gene expression of the considered storage proteins was up regulated shortly after 10 DPA, reaching a maximum between 21 and 28 DPA (Figure 3-Figure 8). This is in accordance with results from Wan *et al.* (2008), showing huge changes in the expression for many genes in the switch from 10 to 12 DPA and again from 21 to 28 DPA. The results also agree with gene expression patterns of storage protein families described by Noma *et al.* (<sup>5</sup>). At N100, an additional foliar N application at anthesis increased the gene expression of all tested storage proteins except LMW-GS. This agrees with the fact that the proportions of low and medium S containing proteins, such as  $\omega$ -gliadins and HMW-GS, increase and those of high S proteins (LMW-GS) remain the same or decrease due to a higher N supply.<sup>21-25</sup> The results of this experiment

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demonstrate that an additional N application at anthesis stimulated gene expression of the majority of storage proteins, but this effect was only observed when the N level was low. The results from gene expression analysis matched those from SDS-PAGE. The change of total amounts of storage protein fractions as well as their proportions was strongest between 10 and 14 DPA (Figure 9). The later maximum of total amounts at 35 DPA in comparison to the gene expression maximum (21-28 DPA) may be explained by posttranslational modifications during the maturation process.<sup>26</sup>

It is known that wheat storage proteins are accumulated mainly in the outer layers of the starchy endosperm near the aleurone layer<sup>12,27</sup>, which can be confirmed by the results of this experiment. The negative gradient of protein concentration within the grain was shown to be greater when plants grew under both high post-anthesis temperature and high N supply.<sup>13</sup> In this study, it is demonstrated for the first time that a foliar N application at anthesis also affects the distribution of protein bodies within grain cross sections (Figure 10). Due to a foliar N application at anthesis, the percentage of proteins in the sub-aleurone cells increased, while the percentage in the centre of the lobes (starchy endosperm) decreased. These alterations of the protein gradients within a grain will affect the recovery of protein in white flour during the milling process.<sup>28</sup> Consequently, some parts of the grain that were enriched with protein bodies due to the foliar N application at anthesis may be lost during the milling process. However, as the protein enrichment was present in endosperm cells as far as 300  $\mu\text{m}$  from the aleurone layer, positive effects on bread making quality may be expected. Zhou et al. (<sup>27</sup>) tested the bread making quality of different pearling fractions of wheat kernels and found that flour from the mid part of the grain was most suitable for bread making. It was also shown that despite a very high content of glutenins in the outer layers of the grain, baking quality of this pearling fraction was poor because the high fibre

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content hindered the formation of the gluten matrix. This knowledge, combined with the novel information about altered protein gradients within the grain due to a foliar N application at anthesis, may be used by the milling industry to adjust the milling processes for wheat grain.

During grain development, protein bodies fuse, forming bigger protein bodies but at the same time, new small protein bodies appear.<sup>28</sup> Thus, protein bodies of different sizes can be detected in a wheat grain cross section. As larger proteins appear to be rich in HMW-GS, and smaller proteins close to the aleurone layer show high contents of LMW-GS<sup>12</sup>, the protein body size distribution within a grain cross section is of importance for the composition of storage proteins. Up to now, only effects of post-anthesis temperature and total N supply on the protein body size distribution have been examined.<sup>13</sup> The analysis of developing wheat grain (21 DPA) using light microscopy, revealed that protein body size differs depending on the genotype, and can be influenced by foliar N application at anthesis (Figure 11). These changes in protein body size distribution are likely to be connected with modified contents of gliadins and glutenin subunits. The general distribution of protein concentration and protein body size found in this experiment confirms the results of a previous study.<sup>13</sup> In addition, it was shown that alterations of the protein body size due to a foliar N application at anthesis strongly depend on the variety, the total N supply and the region of the grain. Thus, a foliar N application at anthesis can be recommended to improve protein quality of bread wheat by ensuring an optimal N supply – especially under dry conditions – and to keep residual soil N contents at the end of the vegetation season low at the same time.

The results of the present investigation demonstrate that an additional N application at anthesis stimulates expression of genes encoding for the majority of storage proteins, when the N level is low. When N supply was high, no such effect occurred. The change of total

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amounts of storage protein fractions as well as their proportions, measured by SDS-PAGE, was strongest between 10 and 14 DPA, which coincides with the up regulation of gene expression at the same time. A foliar N application at anthesis led to steeper gradients of protein body occurrence and hence protein concentrations within the grain cross sections. The protein concentration in the sub-aleurone layer was increased by a foliar N application, whereas in the centre of the lobes the protein concentration was decreased. These changes will affect the protein recovery in white flours, as proportionally more protein might be lost during milling processes. It may be concluded that a foliar N application at anthesis increased the overall grain protein concentration, but as negative protein gradients across the grain increase, this effect might be diminished.

Furthermore, the results of this study show that protein body size differs depending on the genotype, and can be influenced by foliar N application at anthesis. The results also demonstrate that alterations of the protein body size due to a foliar N application at anthesis strongly depend on the variety, the total N supply and the region of the grain.

#### **5. Abbreviations used**

GPC, grain protein concentration; HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits; DPA, days post anthesis

#### **6. Acknowledgements**

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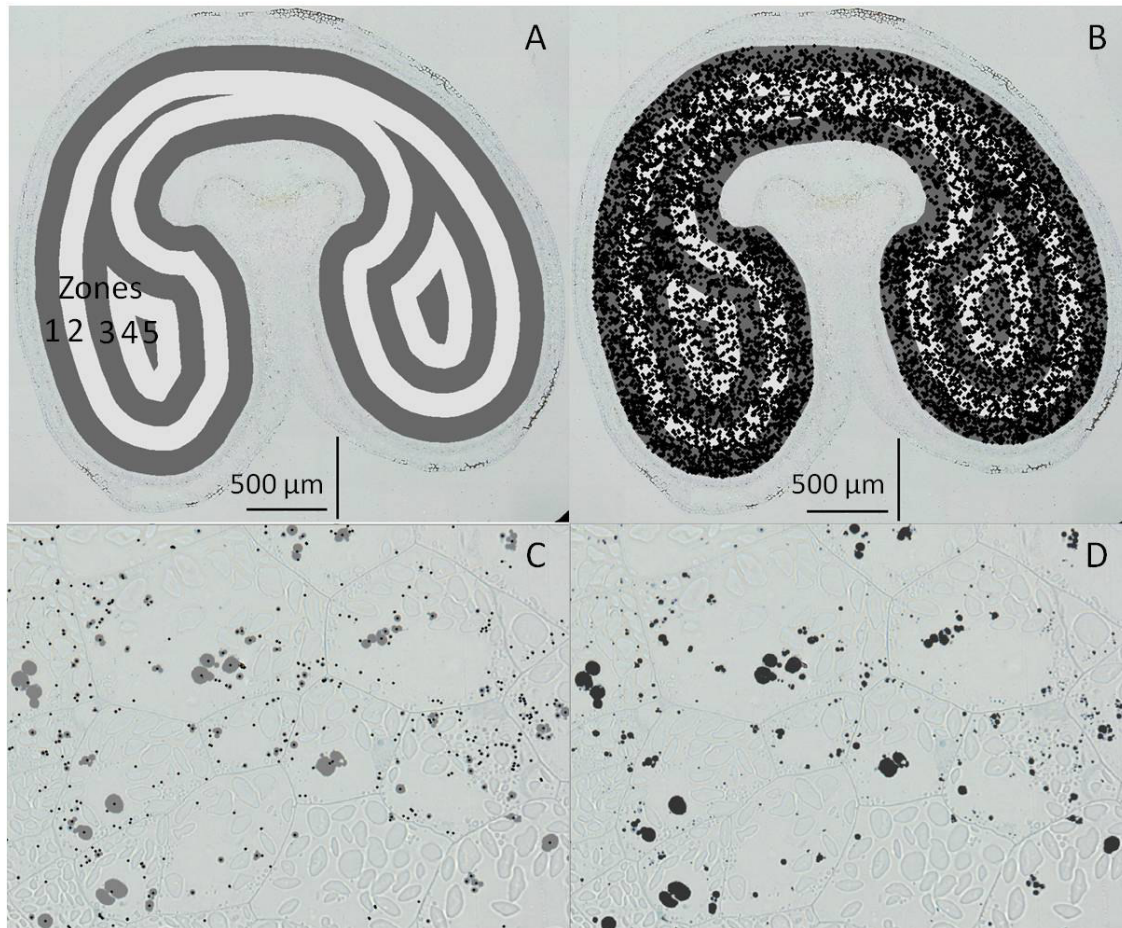


Figure 1: Overview of the grain protein gradient analysis. (A) A composite light-microscopy image of a 1µm thick wheat grain cross section with five concentric zones from the outer to the inner endosperm (labelled 1-5). (B) The zones from image (A) are overlaid on the detected protein bodies. (C) and (D) show the same region of a grain. (C) Detected protein bodies were marked with a central dot. (D) For the analysis of protein body size distribution, the area and distance from the aleurone layer of protein bodies was measured.

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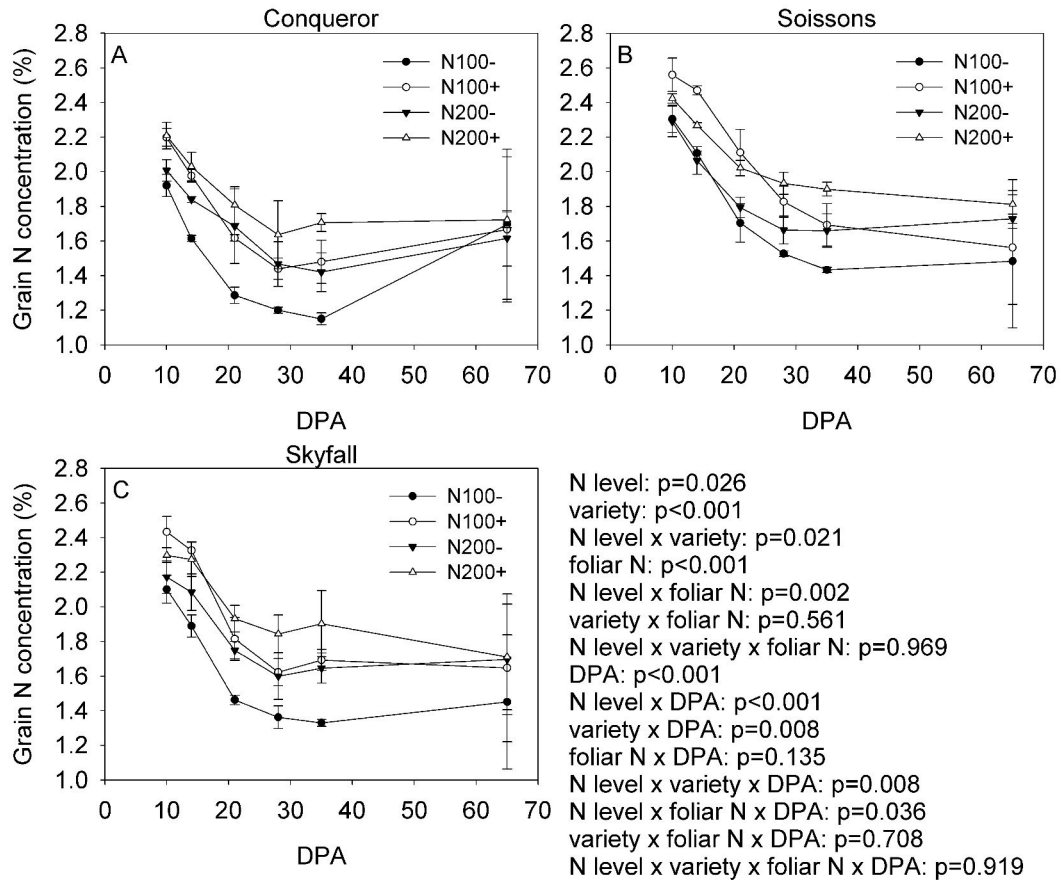


Figure 2: Grain N concentration during grain development (DPA: days post anthesis) as influenced by variety (Conqueror, Soissons, Skyfall), N fertilizer level (N100, N200) and foliar N application at anthesis (+ and - foliar N).

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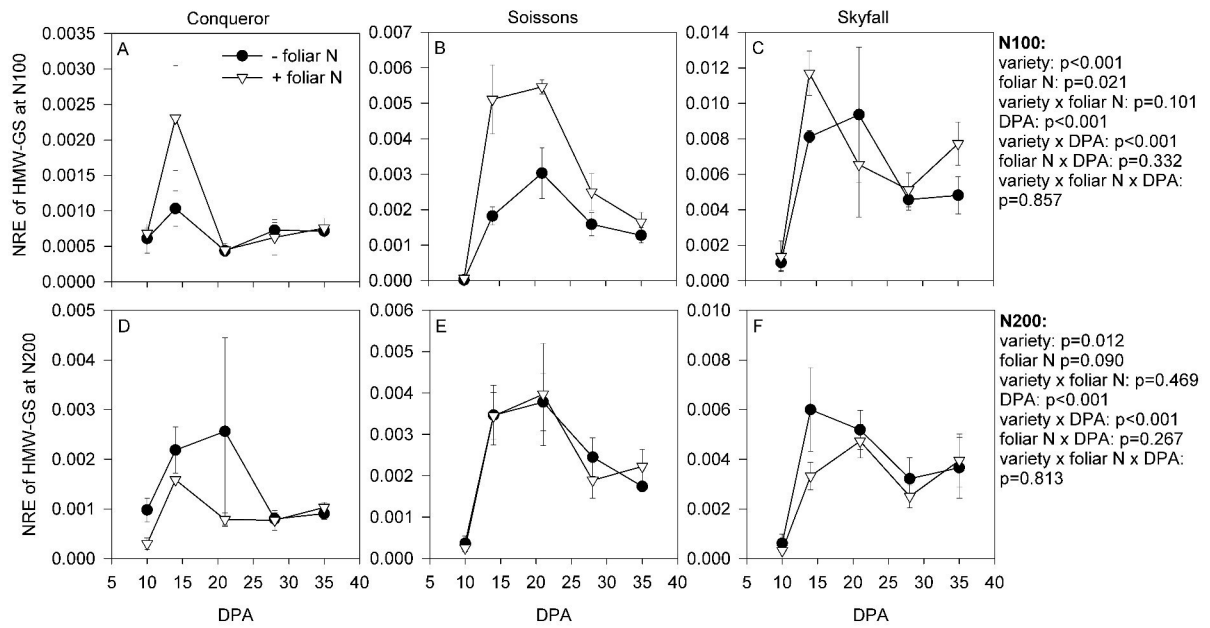


Figure 3: Normalised relative expression (NRE) of the HMW-GS gene family (in arbitrary units) from 10 to 35 days post anthesis (DPA) as influenced by variety (Conqueror, Soissons and Skyfall), N fertilizer level (N100: A, B and C; N200: D, E and F) and foliar N application at anthesis.

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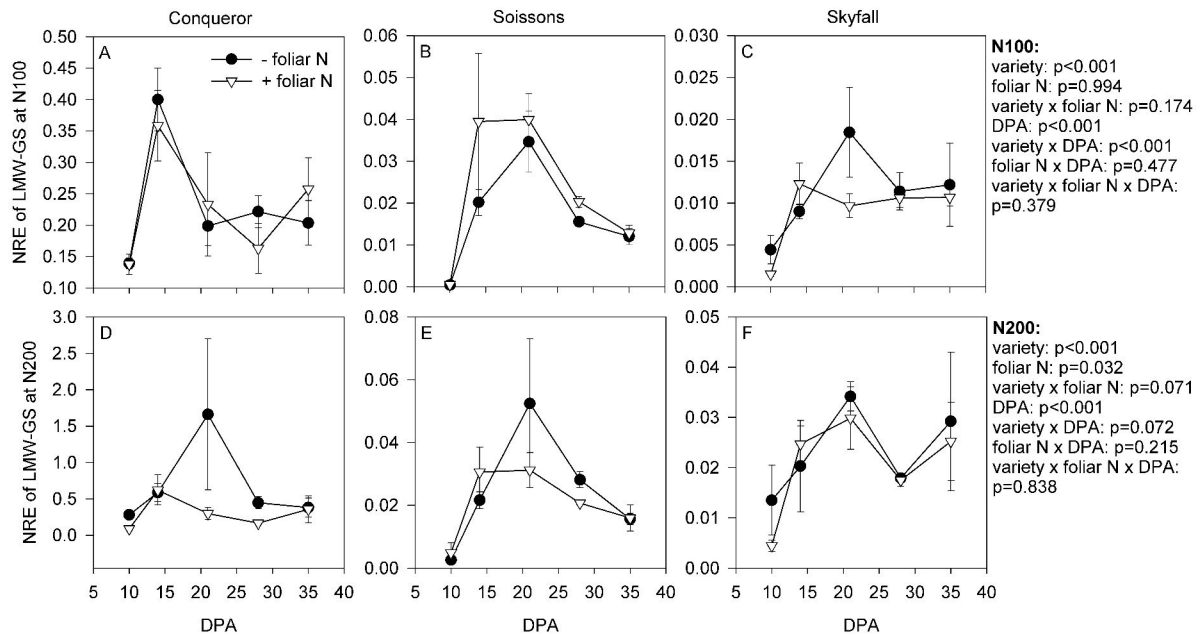


Figure 4: Normalised relative expression (NRE) of the LMW-GS gene family (in arbitrary units) from 10 to 35 days post anthesis (DPA) as influenced by variety (Conqueror, Soissons and Skyfall), N fertilizer level (N100: A, B and C; N200: D, E and F) and foliar N application at anthesis.

#### 4. Effects of a foliar N application on gene expression of protein fractions and protein body distribution

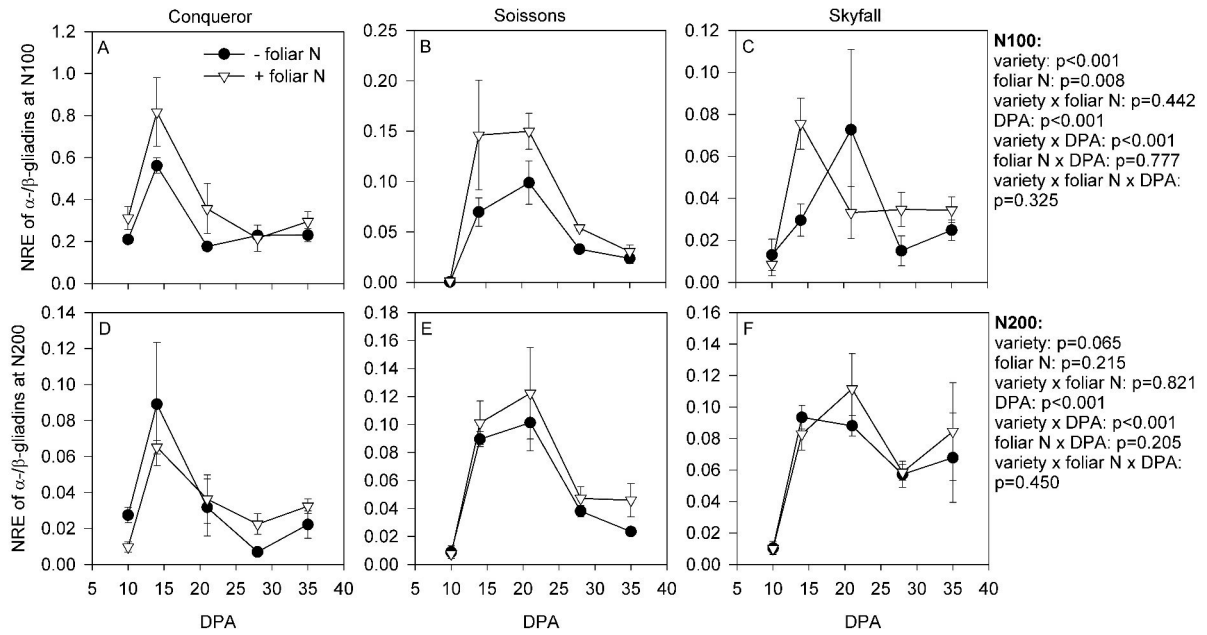


Figure 5: Normalised relative expression (NRE) of the  $\alpha$ - $\beta$ -gliadin gene family (in arbitrary units) from 10 to 35 days post anthesis (DPA) as influenced by variety (Conqueror, Soissons and Skyfall), N fertilizer level (N100: A, B and C; N200: D, E and F) and foliar N application at anthesis.



#### 4. Effects of a foliar N application on gene expression of protein fractions and protein body distribution

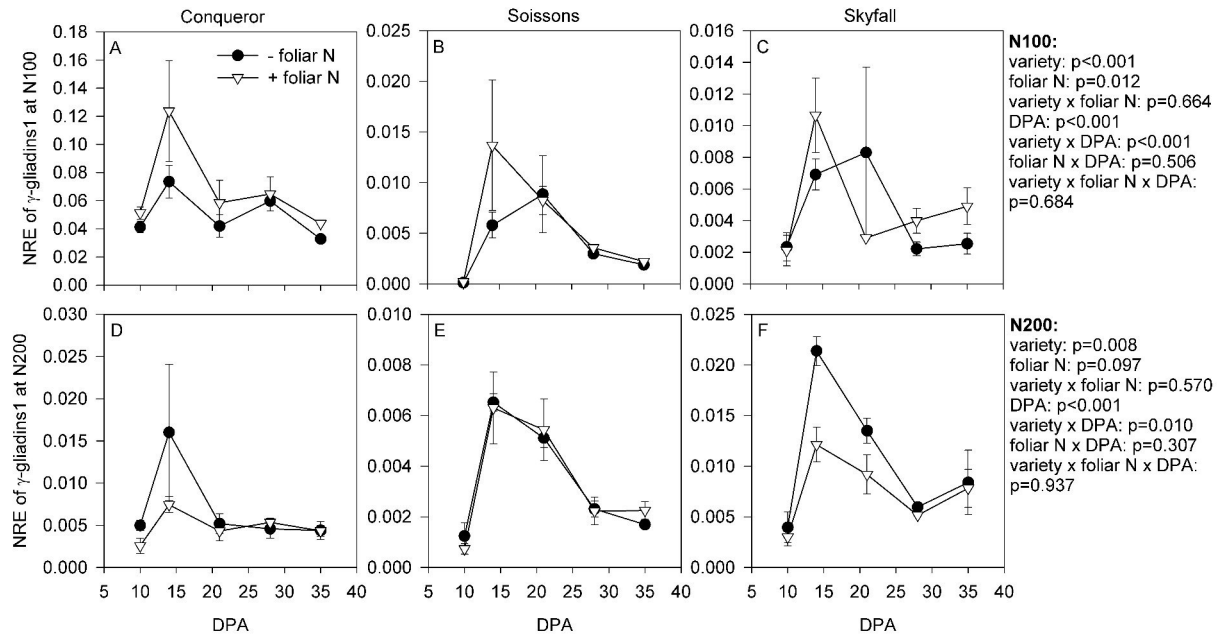


Figure 6: Normalised relative expression (NRE) of the  $\gamma$ -gliadin1 gene family (in arbitrary units) from 10 to 35 days post anthesis (DPA) as influenced by variety (Conqueror, Soissons and Skyfall), N fertilizer level (N100: A, B and C; N200: D, E and F) and foliar N application at anthesis.

#### 4. Effects of a foliar N application on gene expression of protein fractions and protein body distribution

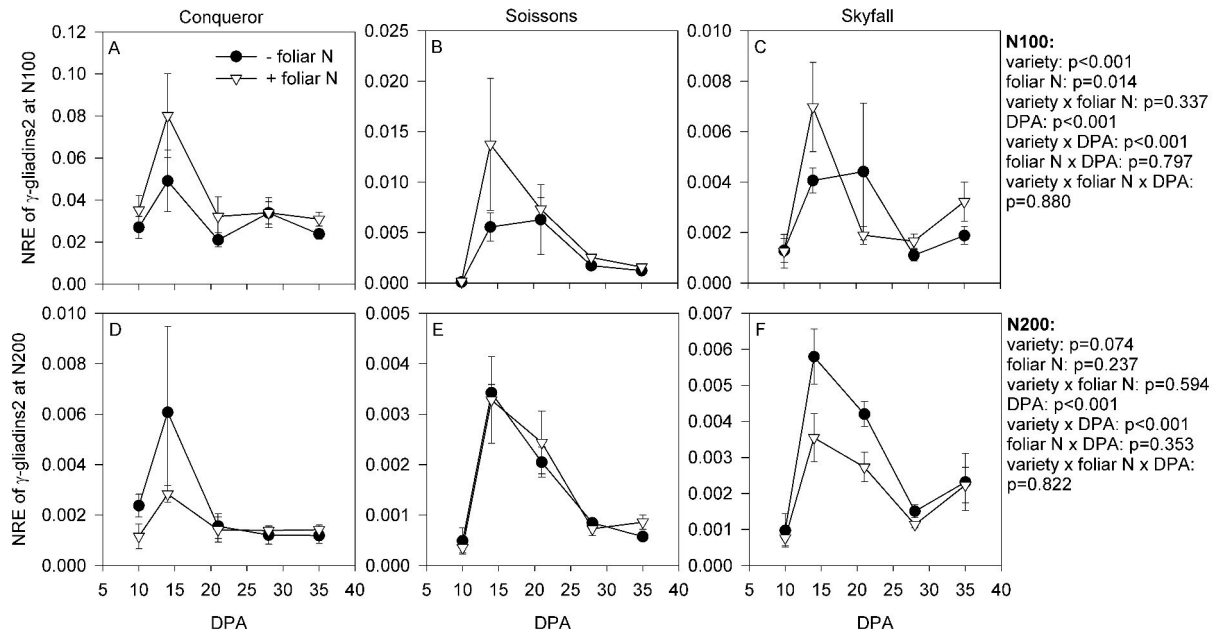


Figure 7: Normalised relative expression (NRE) of the  $\gamma$ -gliadin2 gene family (in arbitrary units) from 10 to 35 days post anthesis (DPA) as influenced by variety (Conqueror, Soissons and Skyfall), N fertilizer level (N100: A, B and C; N200: D, E and F) and foliar N application at anthesis.

#### 4. Effects of a foliar N application on gene expression of protein fractions and protein body distribution

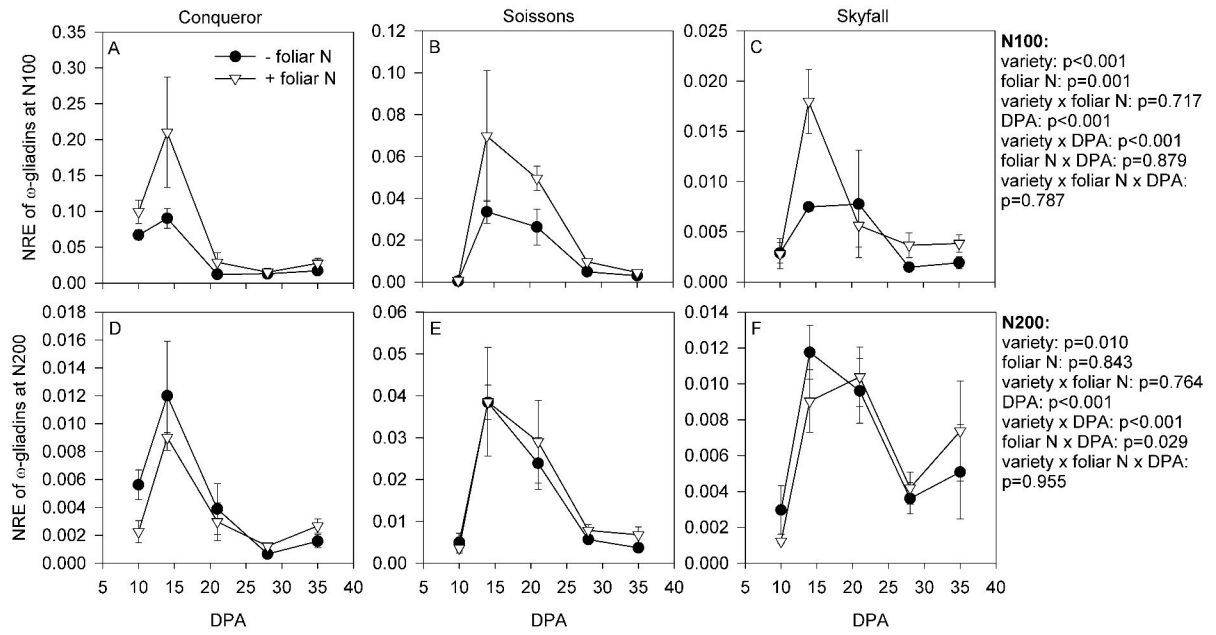


Figure 8: Normalised relative expression (NRE) of the  $\omega$ -gliadin gene family (in arbitrary units) from 10 to 35 days post anthesis (DPA) as influenced by variety (Conqueror, Soissons and Skyfall), N fertilizer level (N100: A, B and C; N200: D, E and F) and foliar N application at anthesis.

#### 4. Effects of a foliar N application on gene expression of protein fractions and protein body distribution

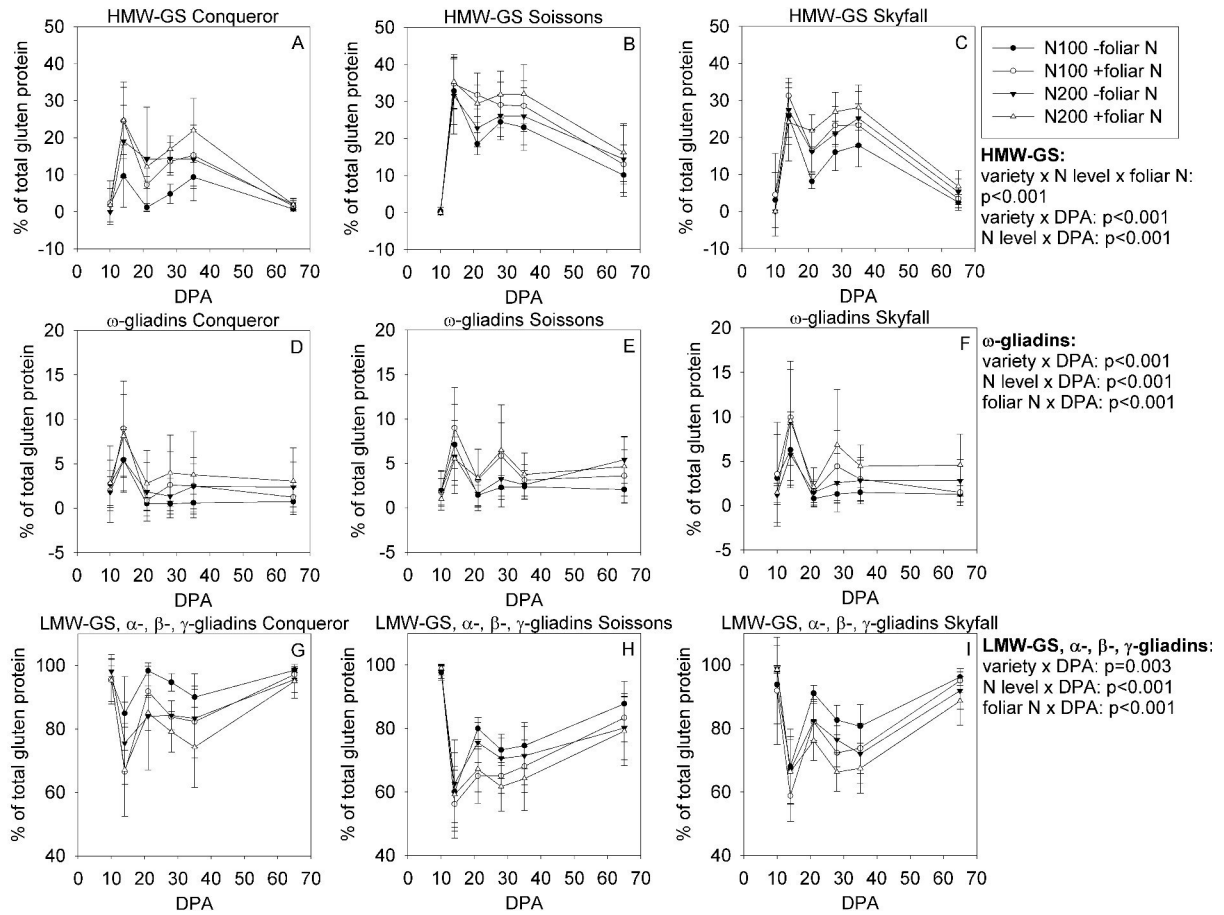


Figure 9: Percentages of storage protein groups during grain development from 10 to 35 days post anthesis (DPA) as influenced by variety (Conqueror, Soissons and Skyfall), N fertilizer level (N100, N200) and foliar N application at anthesis.

#### 4. Effects of a foliar N application on gene expression of protein fractions and protein body distribution

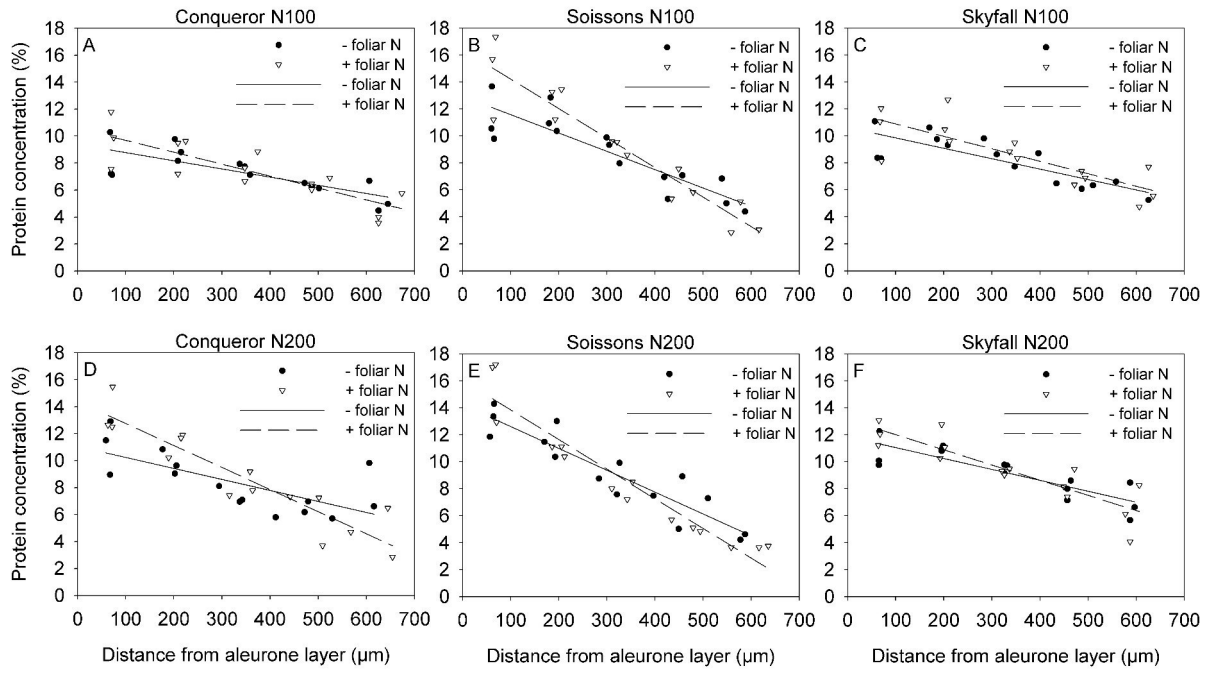


Figure 10: Mean Protein body distribution within grain cross sections and linear predictions as influenced by variety (Conqueror, Soissons and Skyfall), N fertilizer level (N100: A, B and C; N200: D, E and F) and foliar N application at anthesis.

#### 4. Effects of a foliar N application on gene expression of protein fractions and protein body distribution

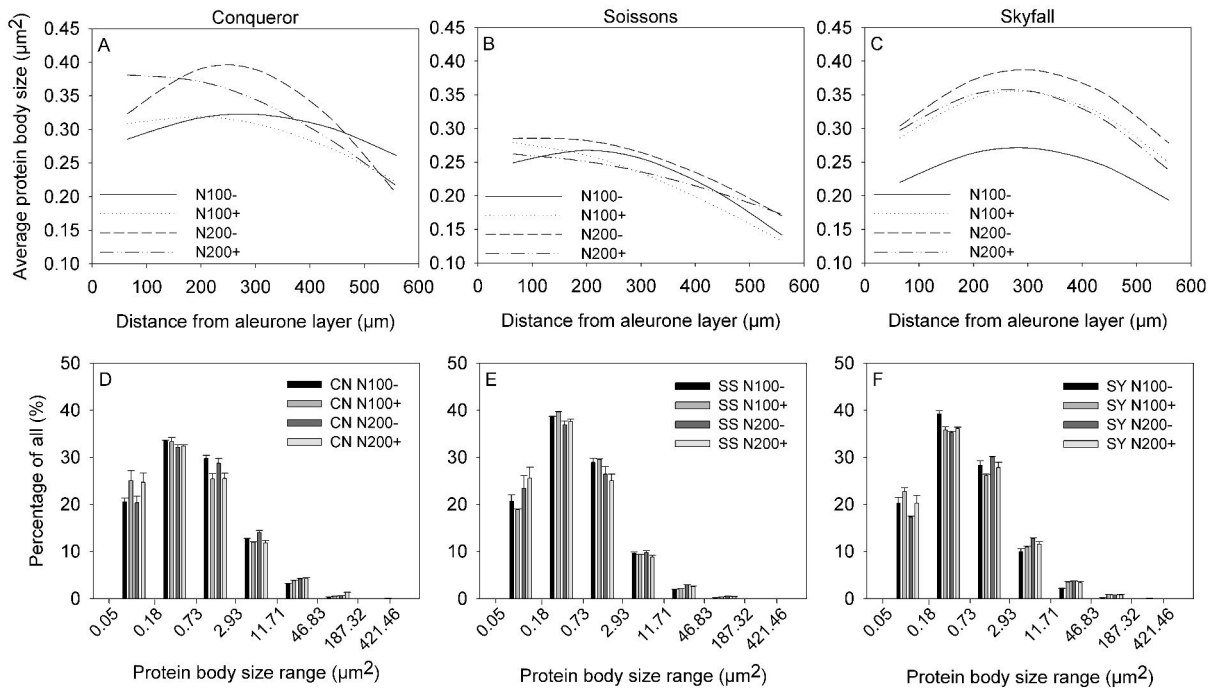


Figure 11: Nonlinear predictions of protein body size distribution with grain cross sections (A-C) and percentages of differently sized protein bodies (D-F) as influenced by variety (Conqueror, Soissons and Skyfall), N fertilizer level (N100, N200) and foliar N application at anthesis.

4. Effects of a foliar N application on gene expression of protein fractions and protein body distribution

Table 1: Primer sequences (forward and reverse) with amplicon size and melting temperature ( $T_m$ ).

Primer	Storage protein family	Sequence (5' to 3')	Amplicon size (bp)	$T_m$ (°C)
18Ssqas2	Reference gene	CTG GTC GGC ATC GTT TAT GGT TG	125	64.6
18Srts1		CAA CTG CGA AAG CAT TTG CCA AGG		65.2
28S-Ribo-rtFor1	Reference gene	GTT TAC GGC GAT GTT AGG AAG TC	95	62.9
28S-Ribo-rtRev1		AAC CGT TTC CAA GGT TGA CAG GC		64.6
TaHMW-ABrtFor2	HMW-GS	CAC AGC TGC CGR CAA TGT GYC	81	63.2-67.0
TaHMW-ABrtRev2		CCA TGC RAG YTG CAG AGA GTT C		62.1-65.8
TaLMW-Glut-abFor3	LMW-GS	GAA GAC CTT CCT CRT CTT TGC C	96	62.1-64.2
TaLMW-Glut-abRev3		TGG TCT CTC CAA ACC AGG GAT G		64.2
Ta-abGliaFor3	$\alpha$ -/ $\beta$ -gliadins	ATG AAG ACC TTT CTC ATC CTT GCC	92	63.6
Ta-abGliaRev3		TGT GGC TGC AAT TGT GGC ACT G		64.2
TaGlia1For	$\gamma$ -gliadins 1 and 2	CCT GCG GCC ACT ATT TCA GCT C	126	65.8
TaGlia2For		GCA GCA ACA GGT GGG TCA AGG T		65.8
TaGlia1/2Rev		GGG ACA TAC ACG TTG CAC ATG G	132	64.2
Ta-OmegaGliaFor1	$\omega$ -gliadins	TGT CCT CCT TGC CAT GGC GAT G	80	65.8
Ta-OmegaGliaRev1		TGA GGT GAT TGT ARC TCT TTG TTG C		62.5-64.1

## Chapter 5

# Effects of a late N fertiliser dose on storage protein composition and bread volume of two wheat varieties differing in quality

*Rossmann, A., Ritter, A., Zwingelberg, M., Scherf, K. A., Rühl, G., Greef, J. M., Mühling, K. H.*



# Effects of a late N fertiliser dose on storage protein composition and bread volume of two wheat varieties differing in quality

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## **Abstract**

Wheat is an important source of energy and protein for humans all over the world and is mainly consumed in form of baked goods, for which a high baking quality is required. The main parameter for bread-making quality predictions of wheat flours is the grain protein concentration (GPC). Therefore, the GPC determines the value of the harvested wheat. Unfortunately, the GPC appears to be no longer an appropriate parameter for baking quality evaluation, given that, especially for high protein varieties, the correlation between GPC and bread volume is poor. A late N application is commonly used to increase GPC and enhance the bread-making quality of wheat flours. To minimise the environmental problems involved in the late use of N fertilisers, it is important to know how much N is necessary to achieve the desired effects. Therefore, in this study, the effects of two different doses of late N application on the GPC, as well as storage protein composition and bread volume were tested in two wheat varieties from different quality groups under field conditions in Germany. The findings of this experiment demonstrate that a low late N application seems to be sufficient to achieve a good baking quality of wheat flours. These results confirm the presumption that GPC is not a suitable parameter for bread-making quality predictions. As the relationships between the single storage protein fractions and bread volume were poor, it can be concluded that only the combined alterations within all gluten protein fractions explain the rise in bread volume.

## 1. Introduction

Wheat is an important source of energy and protein for humans all over the world. As wheat flours have the unique properties to form a strong and elastic dough, the majority of wheat is consumed in form of baked goods and a high baking quality is required. The bread-making quality of wheat flours is being predicted using the grain protein concentration (GPC) as the main parameter. Therefore, the GPC determines the value of the harvested wheat. Unfortunately, the GPC appears to be no longer an appropriate parameter for baking quality evaluation<sup>1-3</sup>. Especially for wheat lots with high protein content, the relationship between GPC and bread volume is poor<sup>4</sup>. As long as no better parameter has been found, it is necessary to consider not only GPC but to examine the composition of storage proteins and bread volume as well when assessing bread-making quality. In Germany, wheat varieties are classified into one of four quality groups on the basis of a number of parameters. Wheat varieties from group E (elite wheat) have excellent bread-making properties, while groups A (quality wheat) and B (bread-making wheat) contain varieties with good and standard baking quality, respectively. Wheat varieties with low baking quality are combined in group C (wheat for other uses) and are preferably used for cookies, fodder and bioethanol production<sup>5</sup>. As GPC is one of the most important criteria, two wheat varieties showing the same level of bread volume may be assigned to different quality groups according to their GPC. By this system, some varieties are favoured and others disadvantaged. Furthermore, farmers are encouraged to prefer high protein varieties which require a higher input of N fertilisers.

A late nitrogen (N) application is a useful method to increase GPC and enhance bread-making quality of wheat flours<sup>6-9</sup>. The influence of different N fertilisation regimens on grain protein composition and baking quality has been investigated before<sup>6,10-12</sup>. Schulz et al.

## 5. Late N fertiliser dose effects on grain protein composition and baking quality

(2015) suggested that one application of N is sufficient to produce wheat of high yield and quality, but in that study, only GPC was measured as a parameter for wheat quality. To minimise the environmental problems involved in the late use of N fertilisers, such as nitrate leaching into the ground water and nitrous oxide emissions, it is of importance to know how much N is necessary to achieve the desired effects. Therefore, taking all these issues into account, in this study, the effects of two different doses of late N application on the GPC, as well as storage protein composition and bread volume were tested in two wheat varieties from different quality groups under field conditions in Germany. The combined investigation of GPC, storage protein composition and bread volume in the current study is novel and may contribute to the challenge of finding a new, quickly and easily detectable parameter for baking quality predictions.

## 5. Late N fertiliser dose effects on grain protein composition and baking quality

### 2. Material and Methods

#### 2.1. Plant cultivation and sample preparation

The two winter wheat cultivars JB Asano and Rumor were grown in a two year field trial under three N fertilisation treatments in Sickte (Lower Saxony, Germany). The two chosen cultivars belong to different wheat quality groups<sup>5</sup>. JB Asano has a high protein concentration and is classified into group A (quality wheat), whereas Rumor with a lower protein concentration belongs to group B (bread wheat). Because both varieties achieve the same level of bread volume with different percentages of grain protein, they were chosen for this study. Wheat plants were grown under three different N fertilisation levels, shown in Table 1. The GPC was estimated by NIRS (near-infrared spectroscopy).

Table 1: N treatments in the field trial, GS (growth stage)

Year	Treatment		N applications			total N kg N ha <sup>-1</sup>
			GS 00	GS 30/31	GS 45/49	
2014	N1	No late N application	50	100-N <sub>min</sub>	0	150
	N2	Low late N application	50	100-N <sub>min</sub>	60	210
	N3	High late N application	50	100-N <sub>min</sub>	80	230
2015	N1	No late N application	80	100-N <sub>min</sub>	0	180
	N2	Low late N application	80	100-N <sub>min</sub>	60	240
	N3	High late N application	80	100-N <sub>min</sub>	80	260

## 5. Late N fertiliser dose effects on grain protein composition and baking quality

### 2.2. Protein extraction and SDS-PAGE

Grain samples were ground using a Titan laminated mill with a 500 µm sieve (Retsch, Haan, Germany). The total gluten protein fraction was extracted from 10 mg wholemeal flour using 50% propan-1-ol + 2% dithiothreitol (DTT) at 50 °C, the extraction being repeated and the supernatants combined. After freeze-drying, the protein was dissolved in loading buffer (50 mM Tris-HCl (pH 6.8), 2 % SDS, 10 % glycerol, 0.1 % bromophenol blue, 200 mM DTT) and loaded on 7 cm gels for 1D SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The gels were cast one day prior to use containing of stacking gel (0.5 M Tris-Base + 0.4 % SDS, pH 6.8) and running gel (1.5 M Tris-Base + 0.4 % SDS, pH 8.8). Precision Plus Protein™ Standard (Biorad) was applied to each gel as protein ladder. Running conditions were 40 V for 30 minutes, then 80 V for 120 minutes. After completion of the run, gels were fixed in a fixing solution (40 % ethanol, 50 % H<sub>2</sub>O and 10 % acetic acid) for one hour. Afterwards, gels were stained with staining solution (1 Coomassie tablet (GE Healthcare) in 1.6 L 10 % acetic acid) for one hour and later, destained in 10 % acetic acid overnight. Gels were then scanned using an Epson Perfection V700 scanner. Gel image analyses were carried out using the GelAnalyzer 2010a software (gelanalyzer.com). The protein bands were classified into three groups according to Wan et al. (2014) and He et al. (2013). The first group corresponded to the HMW-GS, the second group to the ω-gliadins and the third group combined a mixture of LMW-GS, and α-/γ-gliadins.

### 2.3. Micro baking test

The moisture content of flour samples was calculated from the weights of a given amount of flour before and after drying for 2 hours at 110 °C. Afterwards, 10 g of wholemeal flour (14.0 % moisture) were mixed with 0.2 g NaCl and then used to determine optimal water uptake and dough kneading time by means of a farinograph (Farinograph-E, Brabender GmbH & Co.

## 5. Late N fertiliser dose effects on grain protein composition and baking quality

KG, Duisburg, Germany). An optimal dough development was defined at a dough consistency of 550 Brabender Units (530-570 BU)<sup>16</sup>. For the micro baking test, 10 g of flour (14.0 % moisture) were combined with 0.7 g fresh yeast and 0.1 g shortening. 1 mL NaCl/sucrose-solution (1 % NaCl, 2 % sucrose), 0.3 mL 0.004 M L(+)-ascorbic acid solution and the determined amount of water were added and the dough was kneaded for the optimal time required to reach 550 BU. After kneading, the dough was left to rest at 30 °C and 90 % relative humidity for 20 minutes. The dough was reshaped and rounded before secondary proofing for 40 minutes. Finally, the dough was baked for 10 minutes each at 180 °C increasing to 250 °C on an automated proofing baking line<sup>17</sup>. The bread loaves were weighed after cooling and bread volume was measured by surface scanning, using a Volscan Profiler 300 (Stable Micro Systems Ltd, Godalming, UK).

### 2.4. Statistical analysis

For statistical analyses, the GenStat (17<sup>th</sup> edition, VSN International Ltd., Hemel Hempstead, UK) software was used. Data were analysed by ANOVA, the design structure of the experiment (randomised plots) being taken into account. F-tests were used to check for the main effects and interactions between factors. The least-significant difference (LSD) at 5 % ( $p=0.05$ ) level was used for the comparison of relevant means.

### 3. Results

Two winter wheat cultivars were tested under three N fertilisation treatments in a field trial in Germany. The results for grain yield and GPC are shown in Figure 1. Grain yield was influenced significantly by cultivar ( $p=0.004$ ) and year ( $p=0.019$ ) but not by N fertiliser treatment. The average grain yield was higher for Rumor with  $9.5 \text{ t ha}^{-1}$  than for JB Asano with  $9.1 \text{ t ha}^{-1}$ . In 2014, the average grain yield of  $10.3 \text{ t ha}^{-1}$  was higher than in 2015 ( $8.4 \text{ t ha}^{-1}$ ), although the total amount of supplied N was lower.

Considering GPC, there was a significant ( $p<0.05$ ) difference between both cultivars. GPC of JB Asano was higher than that of Rumor in both experimental years with the difference being greater in 2015 (JB Asano: 12.8 %, Rumor: 11.4 %) than in 2014 (JB Asano: 12.4 %, Rumor: 12.2 %). When no late N was supplied, GPC was lowest (10.2 %). The low late N application increased GPC to 12.8 % whereas a GPC of 13.7 % was observed when a high dose of late N was applied.

The protein yield ranged from  $0.9 \text{ t ha}^{-1}$  when no late N was applied to  $1.4 \text{ t ha}^{-1}$  when the late N application was high (Figure 2). The protein yield was higher for JB Asano only in 2015, when the total amount of supplied nitrogen was higher.

Flour moisture content varied in the two experimental years with flour samples from 2014 being less moist (6.8 %) than those from 2015 (7.4 %) (Figure 3 A). In general flour moisture content was very low. Differences between the two tested varieties were observed as well. While N treatment did not affect flour moisture content in JB Asano, in Rumor flour moisture content was highest when a low dose of late N was applied.

Flour water uptake was measured using a Farinograph and the results are presented in Figure 3 B. Flour water uptake for JB Asano was higher ( $6.5 \text{ mL } 10 \text{ g flour}^{-1}$ ) than for Rumor ( $6.2 \text{ mL } 10 \text{ g flour}^{-1}$ ) only in 2015 (higher total N amount). In the two years, the effect of N



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fertiliser treatment on flour water uptake was different. While there was no influence of N fertiliser treatment on flour water uptake observed in 2014, in 2015 the flour water uptake increased due to a low late N application but a high late N application did not induce further enhancement.

Bread volume and specific bread volume are parameters for baking quality evaluation. Statistical analyses revealed a three-way interaction of the experimental factors year, variety and N fertiliser treatment for both measures (Figure 3 C and D). In 2014, the effect of N fertiliser treatment varied in the two varieties. While the low late N application increased bread volume and specific bread volume for JB Asano, no influence of N supply was observed for Rumor. In 2015, the impact of N fertilisation on bread volume and specific bread volume for the two varieties was alike. Both parameters were improved by a low late N application (N2) but no further improvement due to a high late N application (N3) was monitored. The bread volume and specific bread volume of the two varieties were comparable, with differences at some N treatments being inconsistent. JB Asano showed greater volumes than Rumor when low late N (N2) was applied in 2014 and when high late N (N3) was applied in 2015 but when high late N was applied in 2014, the volumes for Rumor exceeded those of JB Asano.

As baking quality depends on the composition of storage proteins, the impact of N fertiliser treatment on amounts and proportions of gluten proteins is of particular interest. The results from SDS-PAGE analyses are shown in Figure 4. Gluten proteins were grouped into the following three fractions: high molecular weight glutenin subunits (HMW-GS),  $\omega$ -gliadins, and low molecular weight glutenin subunits (LMW-GS) with  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins. In 2014 (medium total N supply), the amount of HMW-GS was only increased by a high late N application whereas in 2015 (high total N supply) the amount of HMW-GS was increased due

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to a low late N application and a high late N application led to a further increase. The same effects were observed for  $\omega$ -gliadins. The amount of LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins was increased solely by a high late N application in both years.

As the GPC increased with increasing N supply (low and high late N application), the proportions of each fraction of the total gluten protein is of importance to evaluate alterations. The proportions of HMW-GS were higher in 2014 (26.5 %) than in 2015 (24.5 %) (Figure 4 B). While Rumor showed higher proportions of HMW-GS in 2014, there was no difference between varieties observed in 2015 (higher total N supply). The effect of N fertiliser treatment on the proportions of HMW-GS differed in the two years. No impact was found in 2014 but in 2015 the proportions of HMW-GS were increased due to a low (N2) as well as a high (N3) late N application. The proportions of  $\omega$ -gliadins did not change due to N supply in 2014 but a low late N application increased the proportions of  $\omega$ -gliadins in 2015 and a high late N application led to a further increase. The proportions of LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins were not altered by N fertiliser treatment in 2014 whereas in 2015 both, a low and a high late N application decreased the proportions of LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins.

Regression analysis revealed that GPC only accounted for 32.7 % of the variation in bread volume (Figure 5). This represents a poor correlation between these two parameters. The correlations between the proportions of different gluten protein groups were also investigated (Figure 6). The proportions of  $\omega$ -gliadins and LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins accounted for one third of the variance in bread volume (33.3 % and 27.5 %, respectively). The proportions of HMW-GS only accounted for 13.1 % of the variation in bread volume. When separated by cultivar, all correlations were slightly better for Rumor than for JB Asano.

## 4. Discussion

The two tested cultivars belong to different quality groups according to the German Federal Plant Variety Office <sup>5</sup>. On the basis of a higher GPC, JB Asano is classified into quality group A (quality wheat), while Rumor with a lower GPC belongs to quality group B (bread wheat). Considering two other parameters, namely flour water uptake and bread volume, the two cultivars show comparable results <sup>5</sup>. Because both varieties achieve the same level of bread volume with different percentages of grain protein, they were chosen for this study.

The grain yield was higher for Rumor than for JB Asano (Figure 1 A). As grain yield and GPC are negatively correlated, the lower GPC in Rumor in comparison to JB Asano is not surprising. This finding also confirms the characterisation of the two cultivars by the German Federal Plant Variety Office <sup>5</sup>. The GPC was increased by low (N2) and high (N3) late N application, with the highest GPC being monitored when the late N application was high (Figure 1 B). As GPC is the main parameter for bread making quality evaluations, the increased GPC should lead to improved bread volume. In this study, no clear effect of the N fertiliser treatment on bread volume was observed but an interaction of year, cultivar and N treatment. While the two cultivars showed different reactions to changing N supply in 2014, in 2015, the bread volume of both cultivars improved due to a late N application with no difference between low and high late N dose (Figure 3 C). Even though GPC was increased by a low late N application (N2) and enhanced even more when the late N application was high (N3), the higher GPC in N3 did not lead to further improvements of the bread volume. These findings demonstrate that GPC is not a suitable parameter for bread volume prediction. This is in line with results from Kazman and Innemann (2010), Thanhaeuser et al. (2014) and Xue et al. (2016a), who also report a poor relationship between GPC and bread volume. Gabriel et al. (2017) also found a poor relationship between GPC and bread volume – especially for

## 5. Late N fertiliser dose effects on grain protein composition and baking quality

GPC greater than 12 %. This finding is also underlined by the poor relationship between GPC and bread volume in this experiment evaluated by linear regression analysis ( $r^2=0.327$ ) (Figure 5). When considering each variety separately, the relationship was somewhat poorer for JB Asano ( $r^2=0.303$ ) than for Rumor ( $r^2=0.393$ ). This also corresponds to the findings of Gabriel et al. (2017) in the way that JB Asano shows a higher GPC than Rumor and thus the relationship between GPC and bread volume is weaker. Békés (2012) suggests a nonlinear relation between GPC and bread volume with a saturation effect at higher GPC, which is supported by the results of the current experiment. That the bread volume was increased by a late application of N regardless of its dosage suggests that a low late N dose is sufficient for the production of high quality bread wheat. Anyhow, this effect appears to be strongly influenced by the total N fertiliser supply, weather conditions and choice of variety.

As bread making quality cannot be assessed by GPC alone, it is of particular interest to investigate the composition of storage proteins. The three storage protein groups separated by SDS-PAGE were affected differently by the N fertiliser treatment. Whereas the total amounts of HMW-GS and  $\omega$ -gliadins showed different dynamics in the two experimental years, the total amounts of the group of LMW-GS with  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins was altered similarly in both years. The differences between the experimental years might be due to increased total N amounts in 2015 compared to 2014. But also weather conditions varied greatly between both years, which can be an additional source of variation. As with a high late N application the amount of extracted gluten protein increased, it is important to analyse not only the total amounts of gluten protein fractions (Figure 4 A) but also their proportions (Figure 4 B). It is known that with increasing N supply the proportions of HMW-GS and  $\omega$ -gliadins increase and the proportions of LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins decrease or remain stable<sup>6,19-22</sup>. In this study these effects could be confirmed for a late dose of additional N

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fertiliser as well. That no effects of a late N application on the storage protein composition were observed in 2014 agrees with unaffected bread volumes detected for Rumor. However, even though the gluten protein composition remained unchanged in 2014, a low late N application did improve the bread volume for JB Asano. It is possible, that small shifts in the abundance of single proteins within one or more protein groups caused this improvement. According to the results from 2015, it can be assumed that the improved bread volume due to a late N application was caused by an increased proportion of HMW-GS and  $\omega$ -gliadins as well as decreased proportions of LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins. Nevertheless, all storage protein fractions showed a poor relationship to bread volume (Figure 6). Therefore it can be concluded that only the combined alterations of all gluten protein fractions explain the rise in bread volume.

## 5. Conclusion

The findings of the present study demonstrate that an increased GPC does not automatically induce a higher bread volume. A low late-season N application appears to be sufficient to achieve a good baking quality of wheat flours. A high late dose of N did further increase the GPC but there was no additional effect on the bread volume. These results confirm the presumption that GPC is not a suitable parameter to predict bread-making quality. From our findings it is plausible that the improved bread volume due to a late N application was caused by an increased proportion of HMW-GS and  $\omega$ -gliadins as well as decreased proportions of LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins. As the relationships between the single storage protein fractions and bread volume were poor, it can be concluded that only the combined alterations within all gluten protein fractions explain the rise in bread volume.

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## **6. Acknowledgements**

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## 5. Late N fertiliser dose effects on grain protein composition and baking quality

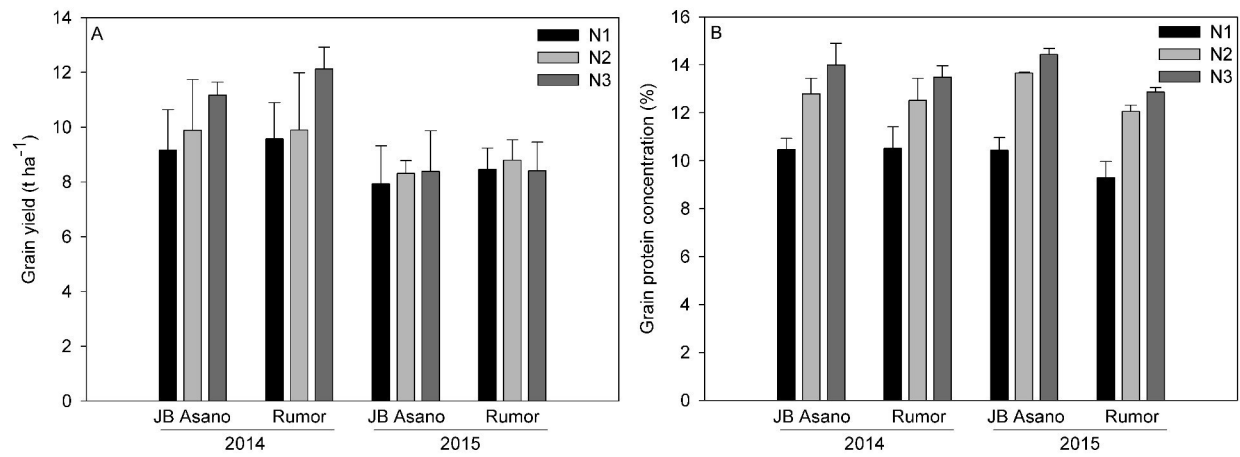


Figure 1: Grain yield (A) and grain protein concentration (B) of two winter wheat cultivars JB Asano and Rumor under three different N fertilisation treatments in two years. Error bars show standard deviations of replicates.



5. Late N fertiliser dose effects on grain protein composition and baking quality

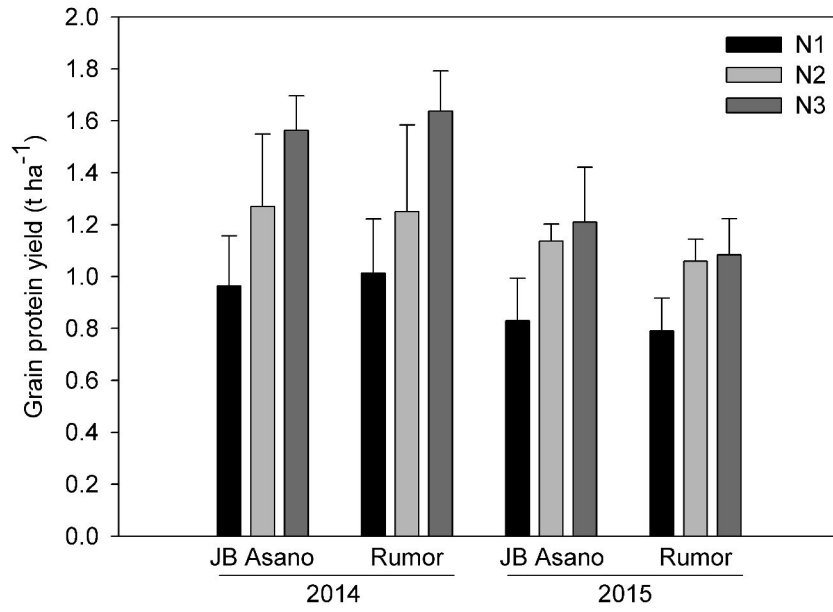


Figure 2: Grain protein yield of two winter wheat cultivars JB Asano and Rumor under three different N fertilisation treatments in two years. Error bars show standard deviations of replicates.

## 5. Late N fertiliser dose effects on grain protein composition and baking quality

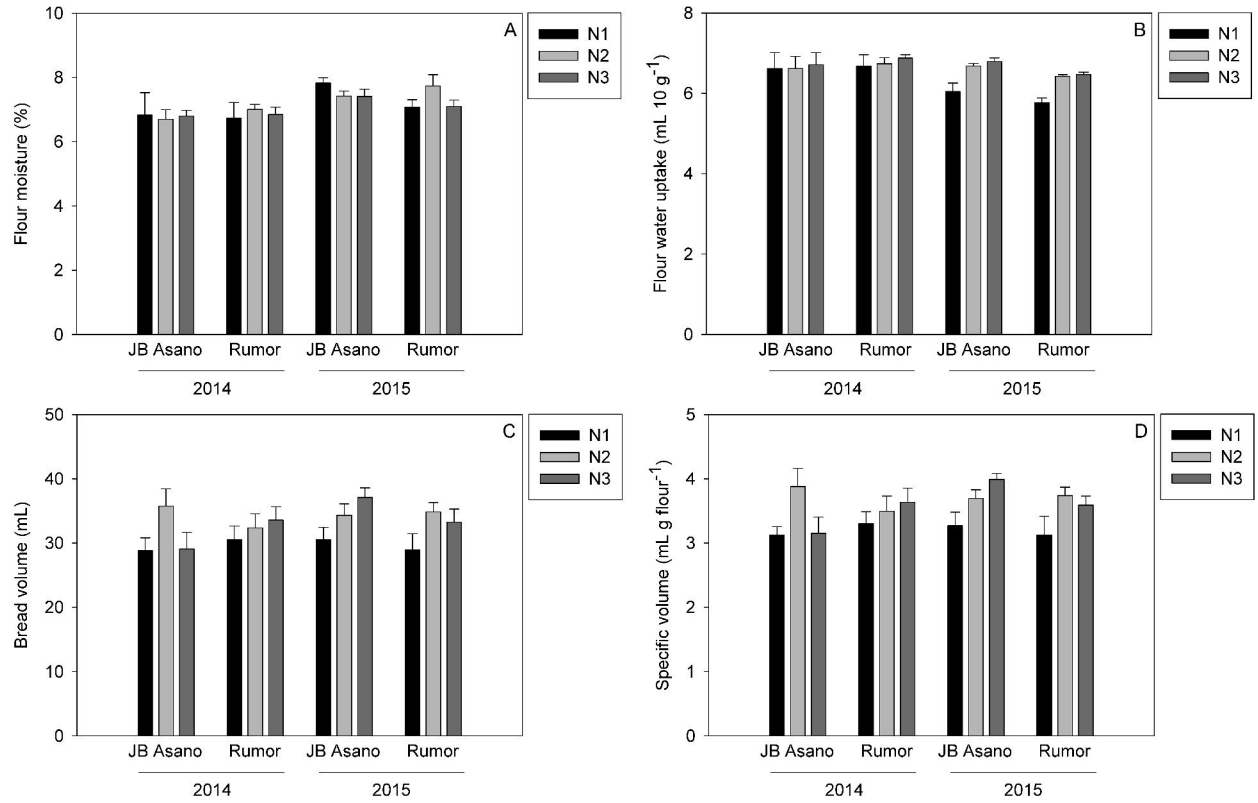


Figure 3: Flour moisture (A), flour water uptake (B), Bread volume (C) and specific volume (D) of two winter wheat cultivars JB Asano and Rumor under three different N fertilisation treatments in two years. Error bars show standard deviations of replicates.

## 5. Late N fertiliser dose effects on grain protein composition and baking quality

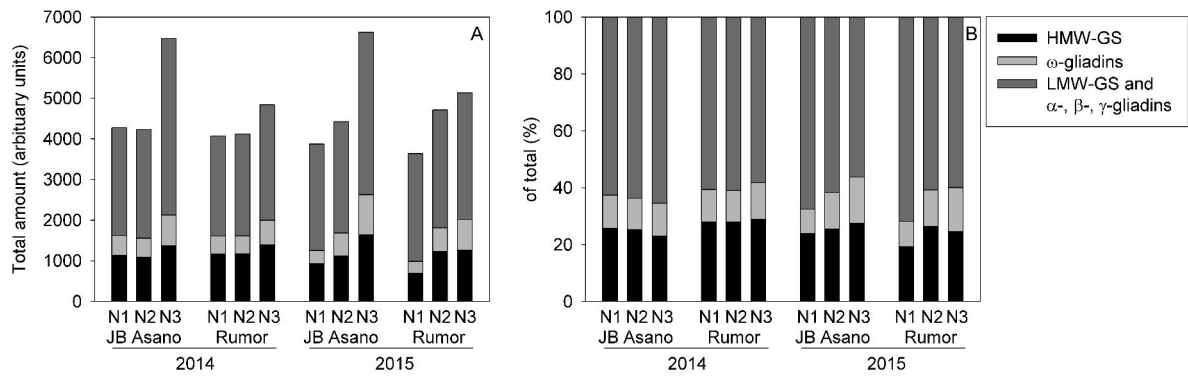


Figure 4: Protein composition measured by SDS-PAGE of two winter wheat cultivars JB Asano and Rumor under three different N fertilisation treatments in two years given in total amounts (arbitrary units) (A) and percentages (B).

5. Late N fertiliser dose effects on grain protein composition and baking quality

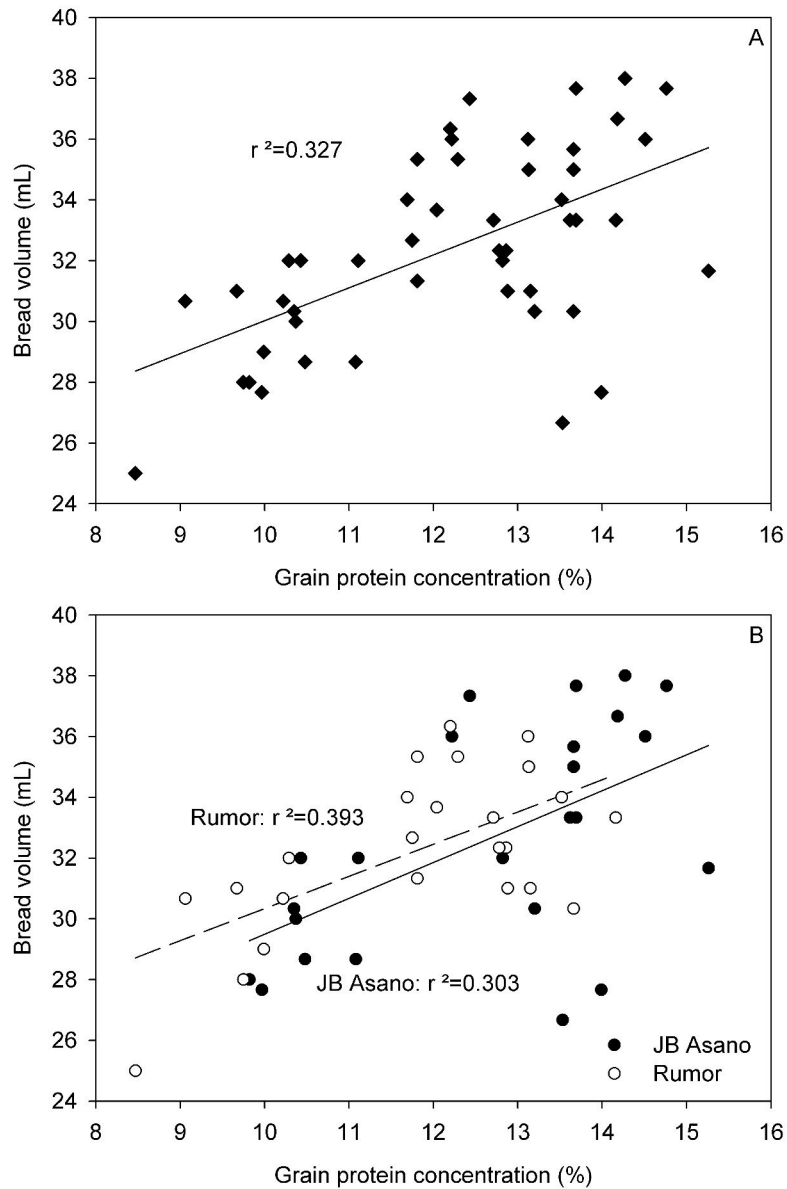


Figure 5: Correlation between grain protein concentration (GPC) and bread volume for the two winter wheat cultivars JB Asano and Rumor (A) and for each cultivar separately (B).

## 5. Late N fertiliser dose effects on grain protein composition and baking quality

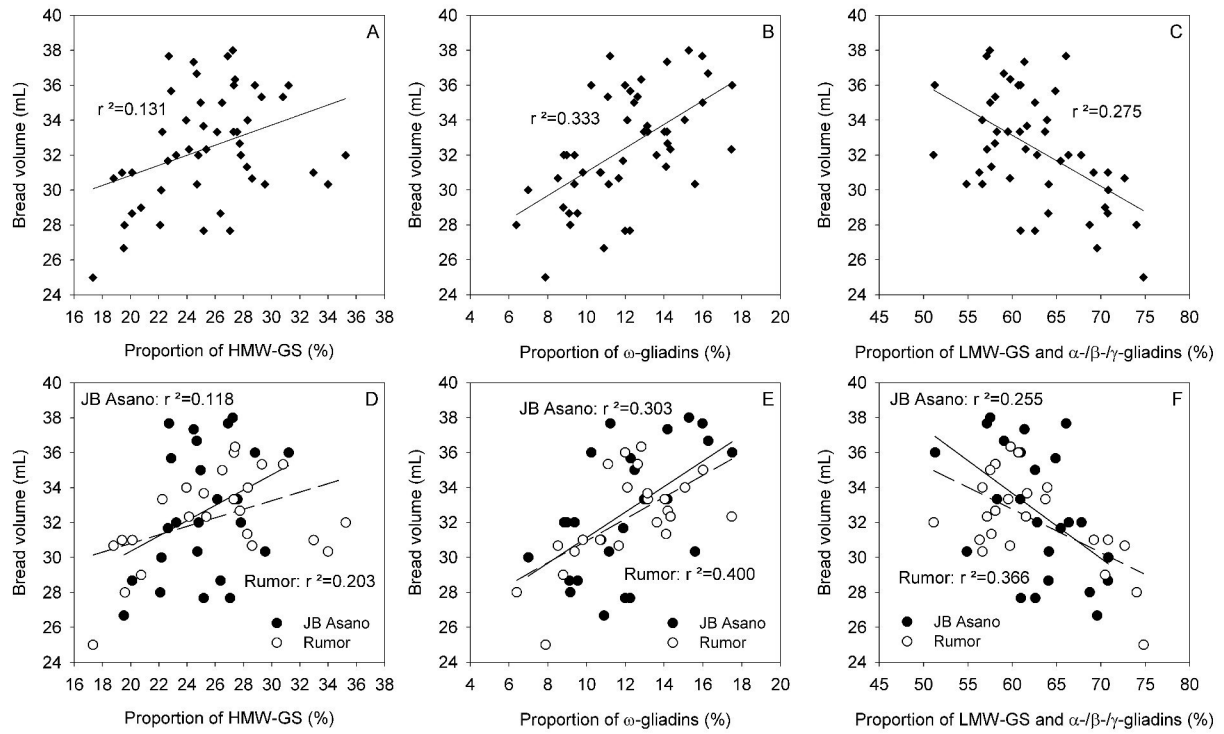


Figure 6: Correlations between HMW-GS (A and D),  $\omega$ -gliadins (B and E) and LMW-GS with  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins (C and F) and bread volume for the two winter wheat cultivars JB Asano and Rumor (A-C) and for each cultivar separately (D-F).

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### 6. General Discussion

Optimal N fertilisation plays an important role in growing wheat and achieving high yields as well as high quality. Farmers usually apply three or four doses of N during the growing season to meet the needs of wheat plants at different growing stages. The late-season N application around flowering time is commonly used to improve the grain protein concentration (GPC) and protein quality. Unfortunately, this late use of N fertilisers can pollute the environment in forms of nitrate leaching or nitrous oxide emissions (Senbayram et al., 2009). To minimise the negative effects on the environment on the one hand and to ensure the production of high quality wheat on the other hand, a re-evaluation of the merits of a late N fertilisation is necessary. Schulz et al. (2015) suggest that a late N fertilisation is outdated and does not improve wheat quality. Unfortunately, in their study, only GPC was measured to evaluate wheat quality. However, recent investigations have shown that in new varieties, the correlation between GPC and baking quality measured by bread volume is poor (Gabriel et al., 2017; Kazman and Innemann, 2010; Thanhaeuser et al., 2014). Therefore, until a more suitable parameter for baking quality prediction has been found, it is important to consider not only the GPC but also examine gluten protein composition and bread volume to adequately evaluate bread-making quality. This work aimed to provide new knowledge about the effects of the late use of N fertilisers in the forms of split, soil or foliar application on the GPC, storage protein composition and bread volume of different wheat varieties. Furthermore, the impact of a late foliar N application on the expression of storage protein genes and the distribution of protein body occurrence and protein body size within the grain was investigated.



## 6. General Discussion

In this general discussion, various effects of a late N application will be discussed based on the results collected in the different experiments. The observations will be brought into the context of the latest research and the results from the different experiments will be approached on a holistic level by relating them to each other.

### **6.1. Effects of a late N application on the grain protein concentration (GPC)**

The GPC is the most important parameter for evaluating the quality of wheat and therefore determines its market value. Nitrogen is an important nutrient, not only for plant biomass production but also for storage protein accumulation in wheat grain.

In the conducted pot experiment as well as in the two field experiments, a higher N fertiliser input led to increased GPCs (Chapter 2, Figure 3 B; Chapter 3, Figure 1 B; Chapter 5, Figure 1 B). This is in agreement with previous studies (Johansson et al., 2001; Raymbek et al., 2017; Wieser and Seilmeier, 1998). An increased GPC was also achieved by splitting the N dose (Chapter 2, Figure 3 B) or applying an additional late-season N dose via the leaves (Chapter 3, Figure 1 B) or via the soil (Chapter 5, Figure 1 B). These findings confirm the results from earlier investigations (Bly and Woodard, 2003; Fuertes-Mendizábal et al., 2010; Gooding and Davies, 1992; Xue et al., 2016a). Generally, the GPC depends on the nutritional status of the wheat plant at the time point of anthesis, which in turn affects the quantity of N taken up during the post-anthesis period (Triboi and Triboi-Blondel, 2002). Kichey et al. (2007) and Taulemesse et al. (2016) found that major proportions of the additional N, absorbed after anthesis, are translocated to the grain and used for grain protein synthesis, which is supported by the results of our study (Chapter 3, Figure 1). When comparing the effects of foliar (Chapter 3) and soil (Chapter 5) application of an additional late-season N dose, the increase of GPC per applied  $\text{kg N ha}^{-1}$  in the late N dose was comparable in both experiments. Anyhow, a foliar N application shows benefits compared with a soil application,

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such as reduced nitrate leaching into the ground water and availability of N despite dry weather conditions. Therefore, to improve the GPC in wheat, an application of a late-season N dose via the leaves should be preferred to a soil application.

The present results demonstrate that even though a late N application increased the GPC, this did not automatically improve the bread volume (Chapter 3, Figures 1 and 5; Chapter 5, Figures 1 and 3). As the correlation between GPC and bread volume was poor (Chapter 3, Figure 8; Chapter 5, Figure 5), it is crucial to examine the composition of grain proteins as well.

### **6.2. Effects of a late N application on the composition of grain storage proteins**

The composition of storage proteins in wheat grains determines the bread-making quality of the flour. The gliadins and glutenins contribute differently to dough rheological properties (Belton, 1999; Goesaert et al., 2005; Khatkar et al., 1995; Zhao et al., 1999; Zörb et al., 2009) and for a high baking quality, a balanced ratio of these storage protein fractions is required. Therefore, the impact of a late N application on the composition of grain proteins is of interest.

In all conducted experiments, the composition of gluten proteins was influenced by a split (Chapter 2), an additional foliar (Chapter 3 and 4) or an additional soil N application (Chapter 5). The split N application and the additional late N application led to increased percentages of HMW-GS. It is known that the variation in HMW-GS is responsible for differences in bread-making quality (Payne et al., 1987) and that the level of HMW-GS is highly correlated with dough development time (Dhaka and Khatkar, 2015). When the late-season N dose was applied via the soil (Chapter 2 and 5), the percentage of LMW-GS was decreased, while it was increased by a late foliar N application (Chapter 3). At first sight, the results from gene expression analysis contradict the increased percentages of LMW-GS, as the gene expression

## 6. General Discussion

for LMW-GS was down-regulated due to a foliar N application (Chapter 4, Figure 3). But this discrepancy can be explained by post-translational modifications during the maturation process (Shewry et al., 2003). For the gliadins, the effect of a late-season N application was inconclusive. While a foliar N application at anthesis led to increased amounts of total gliadins (Chapter 3, Figure 6), the late N application via the soil increased only the percentage of  $\omega$ -gliadins. The increase in gliadins agrees with the stimulated gene expression of all tested gliadin fractions (Chapter 4, Figures 4-7). All these findings support earlier studies showing that the proportions of low to medium sulphur containing proteins, such as  $\omega$ -gliadins and HMW-GS, increase and those of high sulphur containing proteins, such as LWM-GS and  $\alpha$ -/ $\gamma$ -gliadins, remain the same or decrease due to a higher N fertiliser supply (Altenbach et al., 2011; Daniel and Triboi, 2000; Hurkman et al., 2013; Xue et al., 2016b). The small discrepancies between the conducted experiments can be explained by differences in total N supply and weather conditions, which may have affected or blurred the impact of a late-season N application on the composition of storage proteins. Furthermore, the different varieties used in the experiments must be taken into account when comparing the results.

### **6.3. Effects of a foliar N application on the distribution of protein body occurrence and size within the grain**

There are two trafficking pathways of protein bodies with proteins being either aggregated within the lumen of the rough endoplasmic reticulum (ER), or being transported via the Golgi apparatus into the vacuoles (Rubin et al., 1992; Tosi et al., 2009). Two types of protein bodies result from these two pathways. Protein bodies originated in the ER are enriched in HMW-GS, whereas the other protein bodies have high contents of gliadins – especially in young grains (Rubin et al., 1992). The size of protein bodies also indicates which gluten proteins are being accumulated. While gliadins and LMW-GS are more abundant in smaller

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protein bodies close to the aleurone layer, HMW-GS are enriched in bigger protein bodies, which appear in the sub-aleurone layer as well as in the centre of the endosperm (Tosi et al., 2011). In consequence of these relationships, to contemplate the effects of a late-season foliar N application on the distribution of protein body occurrence and protein body size is of interest for wheat producers as well as millers.

In the field experiment conducted in the UK, it was observed that wheat storage proteins mainly accumulated in the outer layers of the starchy endosperm near the aleurone layer (Chapter 4, Figure 10). This confirms findings of earlier investigations (Tosi et al., 2011; Zhou et al., 2018). The effects of two different total N fertilisation levels on the gradient of protein concentration within the grain have been examined lately (Savill et al., 2018). However, in this work we demonstrated for the first time that a foliar N application at anthesis leads to higher percentages of proteins in the sub-aleurone cells, while the percentage of proteins in the centre of the lobes (starchy endosperm) decreased (Chapter 4, Figure 10). These alterations of the protein gradients will affect the recovery of protein in white flour during the milling process (Moore et al., 2016). Consequently, some parts of the grain that were enriched with protein bodies due to the late-season foliar N application may be lost with the bran during the milling process. However, as the protein enrichment was present in endosperm cells as far as 300  $\mu\text{m}$  from the aleurone layer, positive effects on bread-making quality may still be expected. Whilst we cannot comment on the baking performance of white flour, the baking tests conducted with wholemeal flour showed that the bread volume was increased due to a late-season foliar N application when the total N fertiliser supply was low (Chapter 3, Figure 5). This knowledge may be used by the milling industry to adjust the milling process for wheat grain.

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During grain development, protein bodies fuse and form bigger protein bodies but simultaneously, new small protein bodies appear (Moore et al., 2016). Hence, protein bodies of different sizes can be detected in a grain cross section. The analysis of developing wheat grain using light microscopy revealed that protein body size differs depending on the genotype and can be influenced by a late-season foliar N application (Chapter 4, Figure 11). These alterations in protein body size distribution are likely to be connected with modified contents of gliadins and glutenins (Chapter 3, Figure 6) as protein bodies of different sizes accumulate different gluten protein fractions (Tosi et al., 2011). The general distribution of protein body size found in this study confirms the results of previous investigations (Savill et al., 2018). In addition, it was demonstrated that alterations of the protein body size due to a foliar N application at anthesis strongly depend on the variety, the total N fertiliser supply and the region of the grain. Thus, a late-season foliar N application can be recommended to improve protein quality of bread wheat by ensuring an optimal N supply even under dry conditions.

### **6.4. Effects of a late N application on baking quality**

Baking quality of wheat flours is important for the production of foods, such as bread, bread rolls, pizza or cake. Both, the quantity and the composition of proteins influence the baking performance of wheat flours. Especially the gluten proteins are determining baking quality (Goesaert et al., 2005). By forming a continuous network, the glutenin polymers provide strength and elasticity to the dough (Belton, 1999), while the monomeric gliadins act as plasticisers and therefore provide plasticity and viscosity (Khatkar et al., 1995; Goesaert et al., 2005).

In the present experiments, baking quality was estimated by bread volume measurements. It was found that a late-season N application improved bread volumes (Chapter 3, Figure 5;

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Chapter 5, Figure 3). In the first field trial, a foliar N application at anthesis improved the bread volume only when the total N supply prior to anthesis was low (Chapter 3, Figure 5). No such effect was found when the N supply prior to anthesis was high. However, in the second field trial, a late-season N application via the soil improved the bread volume (Chapter 5, Figure 3), even though the N fertiliser level was comparable with the high N supply from the first field trial. It is noteworthy that a low dosage of the late-season N application (via the soil) was sufficient to achieve high bread volumes. Although the high late N application led to a further increase of GPC, it did not cause an additional improvement of the bread volume. In both field experiments, the effects of a late-season N application on the gluten protein composition differed slightly but the effects on bread volume coincide. In the first field trial, the HMW-GS/LMW-GS ratio was decreased and the gliadin/HMW-GS ratio was increased by a late-season foliar N fertilisation when the total N supply was low (Chapter 3, Figure 7). In the second field trial, a late-season N dose led to increased proportions of HMW-GS and  $\omega$ -gliadins, while the proportions of LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins were decreased. The parameter which could explain variance in bread volume in the first field experiment best was the total amount of gliadins (Chapter 3, Figure 8). This is surprising as in previous studies, the levels of HMW-GS and the ratio of HMW-GS/LMW-GS seemed to be the main determining factors of the bread volume (Dhaka and Khatkar, 2015; Payne et al., 1987; Pechanek et al., 1997; Wieser et al., 2000). The results about the impact of the gliadin/glutenin ratio on bread volume in the literature are inconsistent (Dhaka and Khatkar, 2015; Gupta et al., 1992). Wieser and Kieffer (2001) did not find statistical relations between bread volume and the ratios of different storage protein fractions, which agrees with the findings from the second field experiment (Chapter 5, Figures 5 and 6). All these findings demonstrate the complexity and variability of the gluten protein composition and its

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influence on bread-making quality. Until now, the bread volume remains the most direct and reliable parameter to predict baking quality. Yet, to perform the baking tests, numerous steps of sample preparation are needed, which are time consuming and expensive. Thus, the definition of a new reliable and easily assessable indicator for bread-making quality is of particular interest. The results of this work will be useful for future investigations in search of appropriate criteria for baking quality predictions.

### 6.5. Final conclusions

In this work, various aspects of wheat quality and its changeability by late-season N supply were elucidated. In all conducted experiments, a late-season N application increased the GPC but this did not automatically induce a higher bread volume. This confirms the presumption that GPC is not a suitable parameter to predict bread-making quality, which rather depends on the composition and distribution of gluten proteins.

A low (40-60 kg N ha<sup>-1</sup>) late-season N application appears to be sufficient to achieve a good baking quality of wheat flours. From our findings it is plausible that the improved bread volume due to a late N application was caused by an increased proportion of HMW-GS and  $\omega$ -gliadins as well as decreased proportions of LMW-GS and  $\alpha$ -/ $\beta$ -/ $\gamma$ -gliadins.

The findings of this investigation further demonstrate that an additional N application at anthesis stimulates the expression of genes encoding for the majority of storage proteins, when the N level was low. Moreover, a foliar N application at anthesis led to higher protein concentrations in the sub-aleurone layer, whereas in the centre of the lobes the protein concentration was decreased. It can be concluded that a late-season foliar N application increased the overall grain protein concentration, but with negative protein gradients across the grain being increased and thus, relatively more protein being removed with the bran during the milling process, this effect might be diminished. Furthermore, the results of this

## 6. General Discussion

study show that protein body size differs depending on the genotype, and can be influenced by a late-season foliar N application.

The results in their entirety imply that a late-season application of nitrogen can be used to effectively improve baking quality in case the total N uptake was low due to unfavourable weather conditions during growing season prior to anthesis. The findings also show that this effect cannot be observed by measuring GPC alone but also needs a combined inquiry of storage protein composition and distribution as well as bread volume. For the future, a quickly detectable indicator for precise bread making quality predictions still needs to be developed. This work provides new information about the impact of late-season N supply on various grain properties influencing bread-making quality of wheat, which will be useful for future investigations in search of appropriate criteria for baking quality evaluation.



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## 6. General Discussion

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### 7. Summary

Wheat is an important source of energy and protein for humans all over the world. As wheat is primarily consumed in the form of baked goods, baking quality and thus, the concentration and composition of storage proteins are of particular importance. It is common practice to use a late nitrogen (N) application to improve the bread-making quality. However, with N being crucial for protein synthesis on one hand and critical for the environment on the other hand, well established fertilisation practices have to be questioned.

Even with a long history of wheat protein research, the complex interactions between gluten protein composition and dough rheological properties are still not fully understood and were therefore addressed in this Ph.D. work. Moreover, the effects of different N fertilisation regimes – especially a late N application – on storage protein composition, protein body distribution, gluten protein gene expression and baking quality were evaluated.

It was shown that a late N dose generally increased the GPC. However, it was found that the correlation between GPC and bread volume was poor and therefore, GPC should not be used solely to predict bread-making quality. The storage protein composition was altered by a late N fertilisation, regardless whether it was applied via leaves or soil. When the same N amount was split, a late N application led to an increased HMW-GS/LMW-GS ratio, whereas the ratio was decreased due to an additional late foliar N application. Furthermore the results demonstrate that an additional N application at anthesis stimulated expression of genes encoding for the majority of storage proteins, when the N level is low. A late foliar N application also led to steeper gradients of protein body occurrence and hence protein concentrations within grain cross sections, which will affect the protein recovery in white

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flours. The findings of this work show that a late N application improved bread volume only when the total N supply was low. When considering the dosage of the late N application, the results suggest that a dose of 40-60 kg N ha<sup>-1</sup> was sufficient to achieve a good baking quality. These findings confirm that GPC alone is no adequate parameter for baking quality predictions. Furthermore, this work provides new information about the impact of late-season N supply on bread-making quality of wheat, which will be useful for future investigations in search of appropriate criteria for baking quality predictions.

### 8. Zusammenfassung

Weizen ist weltweit eine wichtige Energie- und Proteinquelle in der menschlichen Ernährung. Da Weizen hauptsächlich in Form von Backwaren konsumiert wird, ist die Backqualität und damit die Konzentration und Zusammensetzung der Speicherproteine von besonderer Bedeutung. Landwirte nutzen vor allem die späte Stickstoff-(N)-Düngung, um die Backqualität zu verbessern. Einerseits ist N für die Proteinsynthese unverzichtbar, andererseits birgt diese Praxis jedoch Risiken für die Umwelt. Aus diesen Gründen müssen auch etablierte Düngungsstrategien immer wieder in Frage gestellt und bewertet werden.

Trotz der langen Geschichte der Forschung über Weizenproteine, sind die komplexen Zusammenhänge zwischen der Glutenzusammensetzung und den backtechnologischen Teigeigenschaften, sowie deren Beeinflussung durch Düngung noch immer nicht gänzlich verstanden. Daher sind sie Thema dieser Dissertation. Außerdem wurden in dieser Arbeit Effekte der späten N-Düngung auf die Genexpression von Speicherproteinen und die auf die Proteinkörperverteilung im Korn untersucht und bewertet.

Es konnte gezeigt werden, dass die Proteinkonzentration im Weizenkorn durch eine späte N-Gabe erhöht wurde. Jedoch war die Korrelation zwischen der Proteinkonzentration und dem Brotvolumen nur schwach, weshalb dieser Parameter zur alleinigen Beurteilung der Backqualität unzureichend ist. Die Zusammensetzung der Speicherproteine wurde durch eine späte N-Düngung beeinflusst, wobei sich keine Unterschiede zwischen einer Blattapplikation und einer Düngung über den Boden ergaben. Eine Aufteilung der gesamten N-Menge für die späte N-Gabe führte zu einem höheren HMW-GS:LMW-GS-Verhältnis, während dies durch eine zusätzliche späte N-Gabe reduziert wurde. Weiterhin zeigen die Ergebnisse, dass im Falle einer geringen N-Gesamtmenge die Genexpression der Mehrheit

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der Speicherproteine durch eine späte N-Gabe angeregt wurde. Außerdem führte eine späte N-Gabe zu steileren Gradienten der Proteinkonzentration innerhalb eines Kornquerschnitts, wodurch eine Beeinflussung der Proteinverwertung bei der Herstellung von Weißmehl zu erwarten ist. Die Ergebnisse dieser Arbeit zeigen, dass eine späte N-Düngung das Brotvolumen erhöhte, wenn die gesamte N-Düngermenge gering war. Dabei war eine Applikation von 40-60 kg N ha<sup>-1</sup> ausreichend, um die gewünschten Effekte zu erzielen.

Es konnte in dieser Arbeit bestätigt werden, dass die Proteinkonzentration im Korn allein kein adäquater Parameter für die Beurteilung der Backqualität ist. Außerdem wurden neue Informationen zum Einfluss einer späten N-Düngung auf die Backqualität von Weizen geliefert. Diese bilden eine Grundlage für weitere Untersuchungen im Rahmen der Suche nach angemessenen Kriterien für die Bewertung der Backqualität.

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EMPLOYMENT

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EDUCATION

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08/2017 - 09/2017      Research within the PhD work  
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### ADDITIONAL INFORMATION

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### PUBLIC PRESENTATIONS

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- 08/2017 Rossmann, Anne; Mühling, Karl-Hermann  
**Effects of split nitrogen application on grain protein concentration and composition in winter wheat at different N fertilisation rates**  
International Plant Nutrition Colloquium, Copenhagen, Denmark
- 05/2017 Rossmann, Anne; Buchner, Peter; Hawkesford, Malcolm J.; Mühling, Karl-Hermann  
**Einfluss einer Harnstoff-Blattdüngung zur Blüte auf die Proteinkonzentration und -zusammensetzung im Weizenkorn**  
Annual Conference of Deutsche Gesellschaft für Qualitätsforschung (Pflanzliche Nahrungsmittel) e. V., Geisenheim, Germany
- 03/2016 Rossmann, Anne; Mühling, Karl-Hermann  
**Einfluss einer geteilten N-Applikation unter verschiedenen N-Versorgungsstufen auf die Proteinmenge und -zusammensetzung in Winterweizen**  
Annual Conference of Deutsche Gesellschaft für Qualitätsforschung (Pflanzliche Nahrungsmittel) e. V., Berlin, Germany

### POSTER PRESENTATIONS

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- 05/2017 Rossmann, Anne; Mühling, Karl-Hermann  
**Effects of split nitrogen application on grain protein concentration and composition in winter wheat at different N fertilisation rates**  
Annual Conference of the German Society of Plant Nutrition, Gießen, Germany

09/2015 Xue, Cheng; Rossmann, Anne, Schulte auf'm Erley, Gunda; Mühling, Karl-Hermann

**Split nitrogen application improves wheat baking quality by influencing protein composition rather than concentration**

Annual Conference of the German Society of Plant Nutrition, Göttingen, Germany

PUBLICATIONS

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2016 Xue, C., Schulte auf 'm Erley, G., Rossmann, A., Schuster, R., Koehler, P. and Mühling, K.-H. (2016) 'Split nitrogen application improves wheat baking quality by influencing protein composition rather than concentration', *Frontiers in Plant Science*, 7, pp. 1–11.  
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