





Aus dem Institut für Pflanzenernährung und Bodenkunde  
der Christian-Albrechts-Universität zu Kiel

**Storage protein quality, metabolite composition, and baking quality  
of winter wheat as affected by late sulfur fertilization**

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**Habt Ehrfurcht vor der Pflanze.**

**Alles lebt durch sie.**

*Friedrich Walter Domke (1899-1988)*

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## **Chapter 1**

### **General Introduction**

## 1. General Introduction

### 1.1. Wheat (*Triticum aestivum*) – production, consumption, and genetic background

Besides France, Germany is one of the most important countries for wheat production in Europe. Compared to France that produced 5.4% of world's wheat production in 2007, Germany produced only 2% less. Furthermore, the consumption of wheat is highly important. Germans consumed about 82 kg wheat per capita in 2007, which is about 6-times the amount of maize consumption (FAO, 2009). Compared to high protein crops like legume seeds, wheat only contains about half of the protein concentration. Although wheat grain has a lower protein concentration of about 8-14% (Shewry et al., 2009), the high amount of human wheat consumption leads to wheat protein intake of about 20.4 g per capita per day. Unlike wheat consumption, Germans consume negligible protein amounts of 0.12 g protein per capita per day from pulses (FAO, 2009).

Data about the consumption of bakery products confirm the high prominence of wheat products and its importance for protein intake in human nutrition. In Germany, men consume about 300 g of bread, bakery and cereal products per day. For men this is the largest food group consumed, whereas for women it is the second largest next to fruits and vegetables (Max Rubner-Institute, 2008). Due to the importance of bread and bakery products and its primary impact for protein intake, protein quality aspects are of increasing interest.

There are many factors that influence wheat protein quality: Genetics, environmental conditions and phyto-pathological factors. The evolutionary genetic background of today's wheat cultivars are built by einkorn and emmer. Emmer (*Triticum turgidum*) was developed from the domestication of *Triticum uratu* (AA) and *Aegilops speltoides* (BB). Later, hybridization of cultivated emmer with *Triticum tauschii* (DD) formed today's bread wheat. That is why today's wheat contains a hexaploid genome (AABBDD). The A genome is related to the A genome of einkorn, whereas the D genome is derived from *T. tauschii* (Feldman, 2001). The B genome derives from

*Aegilops speltoides*. This genetic background is the most important factor influencing the synthesis of wheat grain proteins. Wheat grain proteins furthermore are main determinants of the end-use quality.

## **1.2. Importance of sulfur in agricultural wheat production**

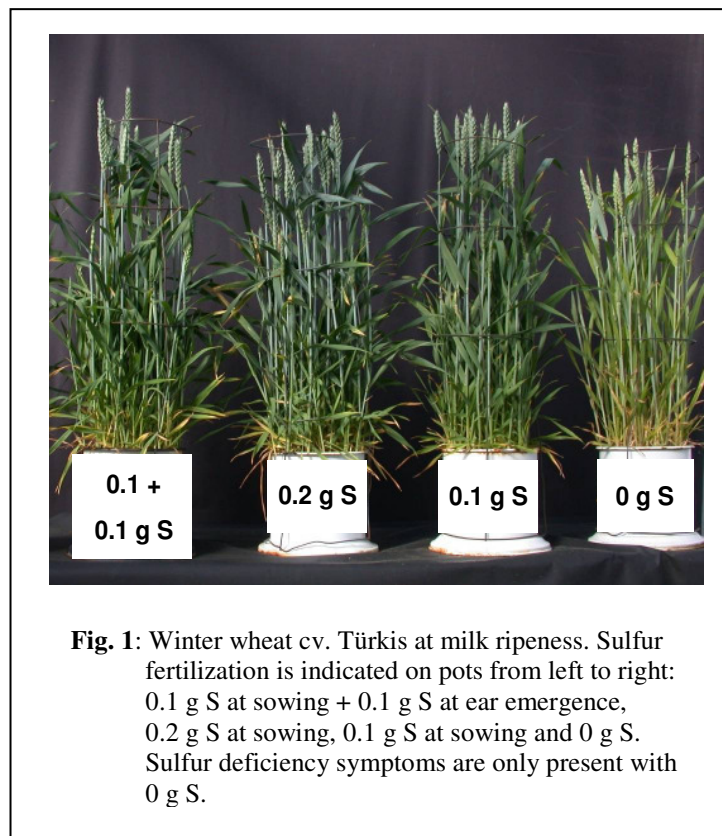
Since wheat is being used for purposes besides human nutrition, wheat breeding during the last decades was concentrating on a magnitude of individual characteristics resulting in the high diversity of wheat cultivars. One example for the breeding success is that today accredited breadmaking cultivars yield about 10-30 dt ha<sup>-1</sup> more than those 40 years ago (Statistisches Bundesamt, 2010).

Furthermore, there are many other factors that influence the protein composition and thus end-use quality of wheat. These factors are environmental conditions like temperature regimens during growth, soil texture, water availability, soil conditions and nutrient supply. In contrast, one factor that may be easily adjusted is an adequate fertilization with essential nutrients. The two elements nitrogen (N) and sulfur (S) are important macronutrients in plant metabolism that predominantly influence protein quantity and its quality. Whereas the impact of N on protein quantity and quality is highly elucidated, the impact of S has just recently become present again.

Sulfur deposition from atmosphere reduced during the last decades due to emission regulations. Between 1990 and 2004, the majority of European countries have reduced their emissions by more than 60% (Vestreng et al., 2007). Furthermore, the use of high yielding cultivars, declining use of S containing fungicides, low S returns of farmyard manure and the use of high-analysis low S fertilizers have enforced the reduced S availability for plants in agriculture (Scherer, 2001). Zhao et al. (1995) examined that 11% of British soils have been of high and 22% of moderate risk for S deficiency. Schnug and von Franck (1985) further observed bulk S depositions in Schleswig-Holstein with 11-20 kg ha<sup>-1</sup> that may not cover S uptake of crop plants. Low bulk S depositions are still present in Schleswig-Holstein as data from two measurement stations show: On the experimental station Lindhof 15.07 kg S ha<sup>-1</sup> and in Schuby 22 kg S ha<sup>-1</sup> were observed in 2007/2008 (Möller, 2010).

In consequence, plants' uptake of S from soil and atmosphere does not cover the requirements to maintain a metabolism without S deficiency symptoms. Sulfur deficiency symptoms were first observed with agricultural crops like canola that have large demands for S. Even though wheat has low demands for S, deficiency symptoms become present and therefore an adequate fertilization management is required. For example during early stages of wheat growth, during stem elongation, critical values for total S were obtained at 1.5 and 1.9 mg g<sup>-1</sup> (Spencer and Freney, 1980; Westfall et al., 1990) and therefore an additional sulfur application is necessary.

A reinforcing effect of S deficiency is the fact that S is hardly translocated within plants. Due to slow SO<sub>4</sub> flow rates in mature leaves that can not compensate for the high relative growth rate, S remains in old leaves instead of being transported into meristematic tissues of young leaves (Bell et al., 1995). Unlike N deficiency, where old leaves are affected, under S deficiency young leaves react with light green brightening (Fig. 1). Whereas N deficiency results in completely reduced growth rate due to decreased protein synthesis, S deficiency does not necessarily become present.



**Fig. 1:** Winter wheat cv. Türkis at milk ripeness. Sulfur fertilization is indicated on pots from left to right: 0.1 g S at sowing + 0.1 g S at ear emergence, 0.2 g S at sowing, 0.1 g S at sowing and 0 g S. Sulfur deficiency symptoms are only present with 0 g S.

Zhao et al. (1996) observed no deficiency symptoms at latent S deficiency. Steinfurth (Steinfurth, 2008) confirmed these findings. Although there was no reduction in grain yield and protein concentration, N/S ratios of 21:1 in wheat flour were detected due to low S application. In general, N/S ratios of higher than 17:1 and S concentration lower than 0.12% in wheat flour are defined as S deficient (Randall et al., 1981). Thus, the so far observed S deficiency may be interpreted as only the tip of the iceberg while a high percentage of low quality wheat is produced without knowledge.

### **1.3. The role of sulfur in wheat plant metabolism**

In general, plants acquire sulfur for several metabolic functions: synthesis of coenzymes, structural components of proteins and enzymes, secondary metabolites and low-molecular-weight-peptides like glutathione. Plant proteins contain about 80% of all organic N and S present in plant dry matter (Dijkshoorn and van Wijk, 1967).

This high percentage in proteins becomes evident since S primarily is a component of the S-containing amino acids, cysteine and methionine. These amino acids are precursors for secondary metabolites, glutathione and phytochelatins and during generative growth also for grain storage proteins (Scherer, 2001). Due to its thiol group (-SH), cysteine has the ability to form disulfide bonds with other cysteine molecules that regulate enzyme activity or build polymers like phytochelatins for the detoxification of heavy metals and polymeric storage proteins. Unlike organic sulfur, inorganic sulfur is further a constituent of prosthetic groups of enzymes that fulfill both structural and catalytic roles like electron transport (Meyer, 2008).

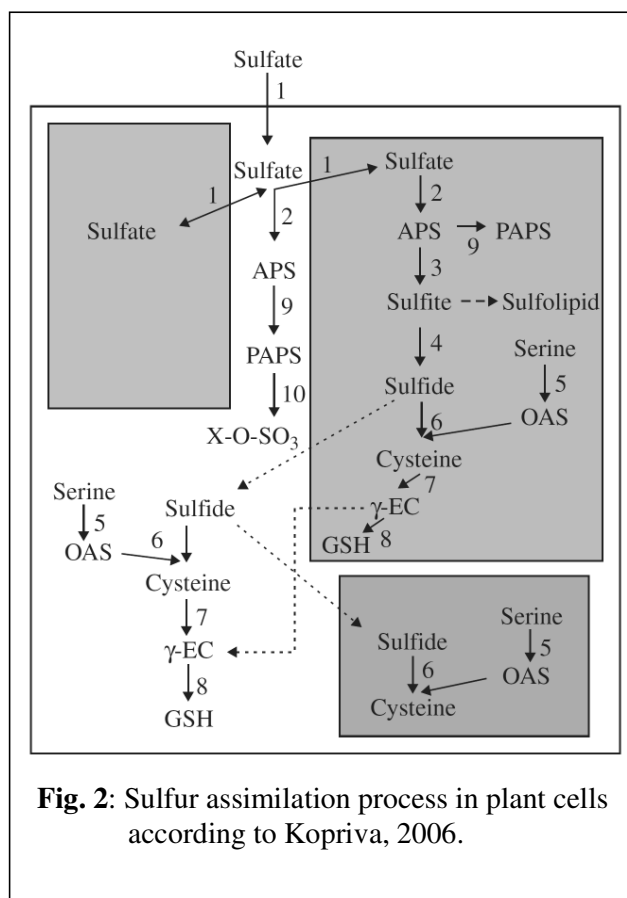
Beside its role as component of phytochelatins, the tripeptide glutathione has several other functions in wheat plant metabolism. As a component of the ascorbate-glutathione-cycle, glutathione detoxifies reactive oxygen species, maintains a strictly regulated redox state within the cell (Noctor and Foyer, 1998) and detoxifies xenobiotics (Schröder et al., 2007). Since the main places of S assimilation are expanding leaves, glutathione is a transport molecule for S via the phloem from the shoot to the root and to generative plant tissues like ears (Rennenberg et al., 1979). During grain development, glutathione is one of the main S-containing compounds that

is supplying S to the grain. Furthermore, grains contain the enzymatic apparatus for glutathione hydrolysis and methionine synthesis from cysteine (Fitzgerald et al., 1999) to synthesize storage proteins.

In storage proteins, cysteine is a primary compound that is needed for the adequate formation of S-rich storage proteins. These proteins and especially gluten proteins are essential for breadmaking processes (Shewry et al., 1994; Wieser et al., 1998). A detailed description of storage proteins and their relation to baking quality is to be found in chapter 1.4.

Plants are taking up S predominantly from soil in form of the anion  $\text{SO}_4^{2-}$  via  $\text{H}^+/\text{SO}_4^{2-}$  co-transporter across the root cell plasma membrane (Clarkson et al., 1993; Buchner et al., 2004). There are two types of sulfate transporters in roots cells: high affinity transporters that are predominantly expressed at S starvation and those of low affinity that are constitutively expressed (Buchner et al., 2004). As a second entrance way, atmospheric sulfur dioxide ( $\text{SO}_2$ ) can enter plant leaves via the stomata and the cuticle (Faller et al., 1970; Fowler and Unsworth, 1974). Previous investigations on cotton showed that S uptake by leaves depends on S supply to the roots, because only 30% of S in the plant derived from  $\text{SO}_2$  from precipitation, whereas under S starving conditions in soil, even an amount of 50% was derived from  $\text{SO}_2$  (Olsen, 1957). Sulfur dioxide is oxidized or reduced in plant cells to  $\text{SO}_4$  or  $\text{H}_2\text{S}$ , respectively, and these are further converted to cysteine and glutathione (Hüve et al., 1995).

However, due to emission regulations this way of S uptake is minimized and plants have to take up most of the S from soil via the roots. Since about 98% of S in soil is bound to organic matter (Freney et al., 1975) or further to inorganic soil particles (Barber, 1995) like Al and Fe, clay minerals and sesquioxides, only a small percentage of  $\text{SO}_4^{2-}$  is available for plant roots. After its absorption,  $\text{SO}_4^{2-}$  is rather transported to leaves via the xylem than assimilated in plant roots. After S assimilation, S-containing metabolites are transported back to the roots and further converted into the individual required metabolites. In leaves, S assimilation takes place in chloroplasts. If more  $\text{SO}_4^{2-}$  is taken up than assimilated, it is stored in vacuoles for later S demands. In chloroplasts,



S assimilation starts with the formation of adenosine phosphosulfate catalyzed by ATP sulphurylase (Fig. 2).

This enzyme transfers  $\text{SO}_4^{2-}$  to ATP, resulting in adenosine phosphosulfate and pyrophosphate. As the chemical equilibrium lies on the pyrophosphate molecule, the pyrophosphatase is splitting this molecule to prevent backward-reaction to ATP. The following reduction of APS to sulfite has been subject of considerable debate. There are two pathways. In the first pathway, APS is reduced to sulfite where electrons are derived from glutathione. In a further reaction step, sulfite is reduced by a ferredoxin dependent reductase to bound sulfide (Wray et al., 1998; Leustek et al., 2000). Sulfide is incorporated into O-acetylserine (OAS) by O-acetylserine(thiol)lyase and the first S-containing amino acid cysteine is formed (Leustek, 1996; Kopriva, 2006).

In the second possible pathway APS is phosphorylated by ATP and 3'-phospho-5'-phosphosulfate (PAPS) is formed. Sulfotransferases transfer sulfo-groups to form other S-containing compounds, mainly secondary plant metabolites such as glucosinolates

that are involved in plant defence against biotic and abiotic stress (Mikkelsen et al., 2002; Klein and Papenbrock, 2004).

Sulfate transporters are highly regulated by the nutritional status of the individual cells (Buchner et al., 2004). To optimize sulfate movements within and in-between sink and source tissues, the expression of sulfate transporters is enhanced by increasing concentrations of O-actylerine (Barroso et al., 1995). O-actylerine is a compound that is accumulating under S deficiency because the first S-containing amino acid cysteine is unable to be synthesized. Furthermore, S-containing compounds such as glutathione or sulfate may function as internal signal that declines the expression of sulfate transporters by negative feedback reaction (Rennenberg, 2001). However, it is not yet completely elucidated which metabolite functions as internal signal.

The translocation of S-containing compounds during vegetative and generative growth is of predominant impact for plant metabolism. The main place of S assimilation is the expanding leaf. Unlike sulfate, that is transported via the xylem from roots to shoot, glutathione is a S-containing tripeptide that is used for S transport via the phloem to supply the roots with assimilated S. During generative growth of wheat this situation is changing, since the developing grain is becoming a sink with higher capacity compared to the roots. In wheat, glutathione (Rennenberg et al., 1979) and the metabolite S-methylmethionine (Bourgis et al., 1999) are transport molecules to be found in phloem sap via the developing grain.

However, the supply of S-containing compounds mainly from the flag leaf is different comparing S starving plants with adequately supplied plants (Fitzgerald et al., 1999). In plants starving from S, sulfate and glutathione pools are too small to cover grain demands for S. As a consequence, cysteine and methionine are recovered from protein degradation in leaves. Although protein degradation provides S-containing amino acids for the translocation to the developing grain, translocated S concentration are low and cause severe changes in grain protein composition.

Furthermore, recent investigations elucidated the allocation of several sulfate transporters in individual tissues of wheat plants. All wheat tissues, including those of



glumes and grain, express sulfate transporters (Buchner et al., 2010). As a consequence, wheat grain is able to uptake both, sulfate via the xylem from the roots and S-containing metabolites like glutathione and S-methylmethionine predominantly from the flag leaf. It is not yet elucidated, if the grain prefers sulfate or the translocated S-metabolites from the flag leaf. Both the anabolic and the catabolic enzymatic apparatus are present in grain. However, it is not yet clear, if sulfate that is applied at ear emergence is directly transported to the grain or first assimilated in flag leaves.

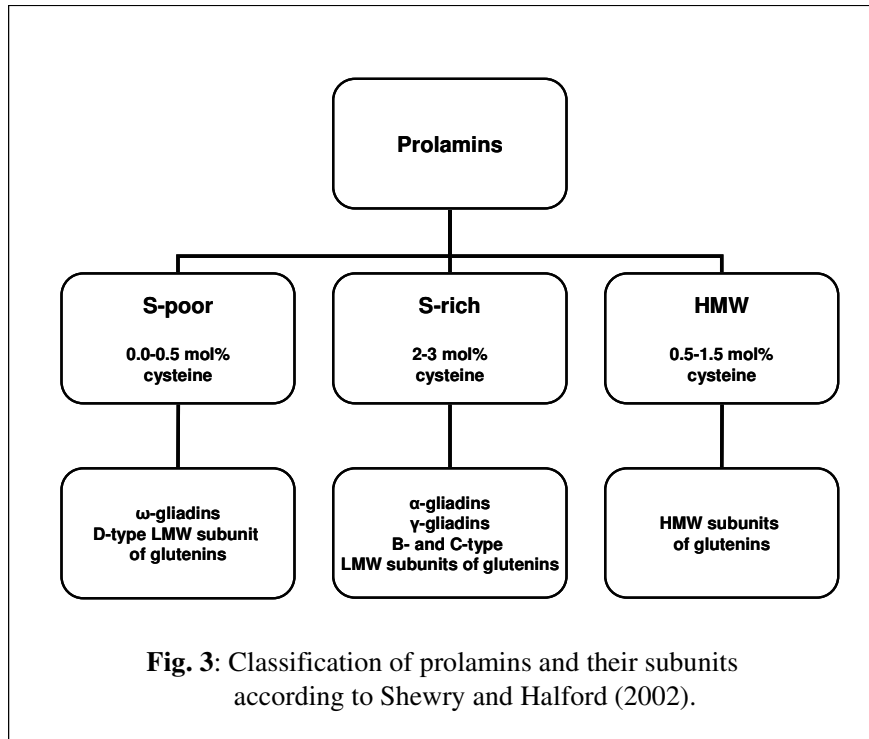
#### **1.4. Composition of storage proteins in wheat**

Storage proteins in wheat are major factors influencing baking quality and are classified according to Osborne (1924) due to their extractability behavior. Albumins and globulins which are mainly metabolic and structural proteins (Goesaert et al., 2005) are extractable with water and NaCl solution. Other storage proteins, the prolamin or gluten proteins are divided into gliadins that are extractable with ethanol, and glutenins that need to be extracted with reducing agents due to their ability to form polymers (Shewry, 1995). Gliadins and glutenins account for about 80-85% of mature wheat grain proteins (Goesaert et al., 2005) and are the main factors that enable remarkable breadmaking properties due to their constitution. Unlike gluten proteins that are only located in the starchy endosperm, globulins are located in both the embryo, the aleurone layer (7S globulins) and in the starchy endosperm (11-12S globulins) (Shewry and Halford, 2002). In wheat 11-12S globulins are called ‘triticins’ and account for about 5% of total seed protein (Singh et al., 1988).

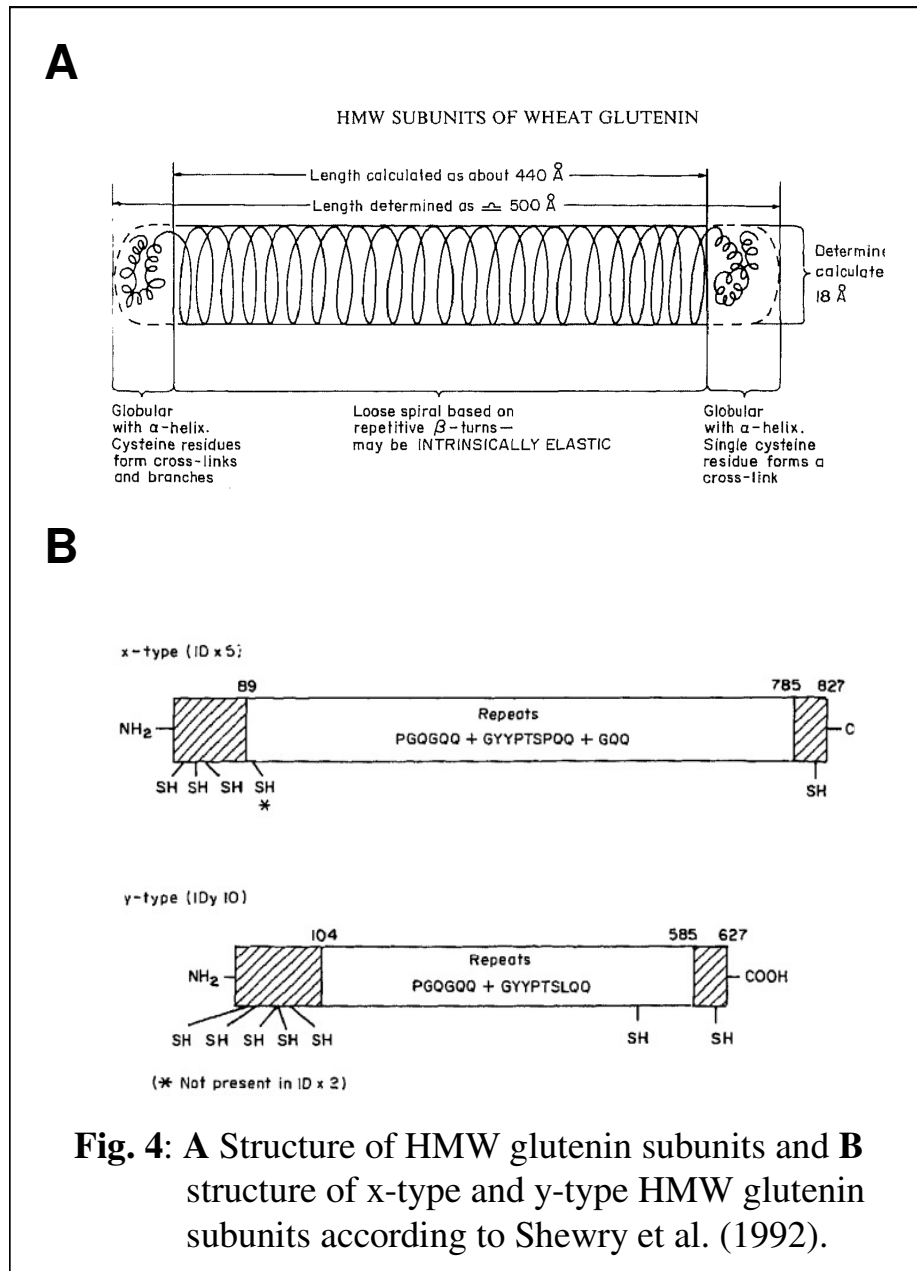
Since the role of globulins in baking quality is limited, this thesis will focus on gliadin and glutenin storage proteins. The synthesis and deposition of gluten proteins in wheat is not yet completely understood. There are hints from storage protein synthesis in maize and rice where prolamins appear to be synthesized within the lumen of the endoplasmic reticulum (ER). This leads to protein bodies that are surrounded by a membrane of ER origin (Coleman et al., 1996).

In wheat, protein bodies are present in both the vacuole and the endoplasmic reticulum (ER) (Rubin et al., 1992). While gliadins are predominantly synthesized in

both types of protein bodies, glutenins are retained in ER-protein bodies. Shewry et al. (2009) further demonstrated that each gluten protein subunit is synthesized at different time during grain development and showed no clear trend. Whereas HMW and LMW subunits were continuously synthesized throughout development, the synthesis of  $\omega$ -gliadins stopped after some time and no further proteins were synthesized.



The basis for dough formation by adding water to wheat flour is the matrix of starch that is surrounded by gluten proteins in the starchy endosperm (Gan et al., 1995). Gluten proteins consist of subunits that either contain no (S-poor), low (High Molecular Weight subunits, HMW) or high (S-rich) S amounts in the form of cysteine (Shewry and Halford, 2002) (Fig. 3). B- and C-type Low Molecular Weight (LMW) subunits of glutenin and  $\gamma$ - and  $\alpha$ -gliadins are S-rich and contain about 2-3% cysteine that is able to form intra- and intermolecular disulfide bonds. D-type LMW subunits of glutenin and  $\omega$ -gliadins are S-poor and contain only about 0-0.5% cysteine. Gliadin proteins are monomeric structures that can only form intramolecular subunits whereas glutenins are able to form both intra- and intermolecular subunits.



HMW and LMW glutenin subunits contain of a loose spiral based on repetitive domain that are bordered by  $\alpha$ -helices each on the N- and C-terminal domain (Fig. 4A). Those  $\alpha$ -helices contain SH-groups of cysteine that are able to form cross-links with other gluten proteins. It is further important to note that HMW glutenins are divided into two types according to their genetic background: y-type HMW subunits that are of low molecular weight and x-type HMW subunits that are of high molecular weight (Shewry et al., 1992). Y-type HMW subunits contain more SH-groups than x-type HMW glutenins (Fig. 4B).

The genetic background of prolamins was highly elucidated by Payne (1987). Since bread wheat is a hexaploid species, there are three different genomes consisting of seven chromosome pairs (A, B, D). Prolamin genes are encoded on all three genomes. HMW subunits occur on the long arm of chromosomes 1A, 1B and 1D, whereas LMW subunits,  $\omega$ - and  $\gamma$ -gliadins occur on the short arms of the same set of chromosomes. In wheat, spontaneous deletion of partial or whole chromosomes is possible. However, those are usually not lethal because of the triplicate genetic information in wheat. In result, it is interesting how S deficiency in wheat deteriorates baking quality and how gluten protein composition is involved.

### **1.5. Effects of sulfur deficiency on wheat quality**

The impact of N and S deficiency on seed yield and quality of wheat flour has already been investigated in various ways (Moss et al., 1981; Haneklaus et al., 1992; Zhao et al., 1999a; Flåte et al., 2005). Reduced N in plants results in declined amino acid and protein synthesis and later in lower seed yield, since N is the main component of proteins. Although N is applied in adequate amounts, S deficiency still leads to disastrous declines in seed yield (McGrath, 1985) and baking quality (Zhao et al., 1999c).

First, seed yield is affected by S deficiency (Randall et al., 1981). However, S fertilization does not continuously increase yield, since it follows a saturation curve as Withers et al. (1997) observed. Only slight increases in yield were observed between 20 and 40 kg S ha<sup>-1</sup> independent from N fertilization, but high potential for yield increase was shown between 0 and 20 kg ha<sup>-1</sup>.

Quality characteristics can still be enhanced although yield is already optimized (Withers et al., 1997). The baking quality or potential of a wheat cultivar is predominantly measured as loaf volume in a baking test. As indirect methods, sedimentation value and dough characteristics like extensibility, mixing properties and resistance are measured. Several investigations confirmed increasing resistance of dough and reduced extensibility due to S deficiency (Kettlewell et al., 1998; Zhao et al., 1999b). As a consequence, increasing the S fertilizer rates resulted in higher loaf

volume due to increased extensibility, lower resistance of dough and better crumb score (Zhao et al., 1999b).

All in all, yield and quality losses due to inadequate fertilization management have drastic effects on the monetary intake of farmers, the bakery industry and human nutrition. As a consequence, N and S fertilization have to be adjusted to soil and weather conditions and plant's nutrient demand. In Germany, N fertilization is typically applied in three dressings: 1<sup>st</sup> in spring at tillering, 2<sup>nd</sup> at stem elongation and a 3<sup>rd</sup> late dressing at ear emergence. The late dressing was observed to increase the gluten protein concentration in wheat grain that is predominantly decisive for baking quality (Jahn-Deesbach and Weipert, 1964).

Sulfur fertilization with wheat is still not yet completely established in Germany. There are soil conditions that cover S demands of plants so that a further S fertilization does not have additional benefit. However, complicated dynamics of organic S compounds make a precise S fertilization difficult (Scherer, 2001). There are as well areas with soil conditions where an S application may benefit yield and quality of wheat. To date, a few investigations show first evidence of a late S fertilization concomitantly with a late N fertilization on baking quality. Seling et al. (2006) and Steinfurth et al. (2008) for example illustrated the impact of late S fertilization on storage protein composition and baking quality. Although no differences in both protein concentration and S concentration were found in wheat grain, protein changes in wheat flour were detected that may be responsible for improved baking quality characteristics (Steinfurth, 2008; Zörb et al., 2009). To date, changes in protein profile were found, however, identification of these proteins is still lacking as well as its individual effect on baking quality.

## **1.6. Objectives**

The objective of this investigation was to examine the impact of a late S fertilization on storage protein composition and baking quality. For this purpose, the whole cascade beginning with S fertilization, S translocation within wheat tissues, storage protein synthesis in wheat grain during development and the resulting baking quality was

examined. Pot experiments with two wheat cultivars differing in baking potential, Batis and Türkis, were used with increasing S fertilization (0 g S as S deficiency, 0.1 g S as moderate S and 0.2 as high S supply at sowing). Batis and Türkis were used for these experiments, since they do not differ in protein concentration and dough characteristics, but in loaf volume. In particular, a late S fertilization with 0.1 g S at sowing and additional 0.1 g S at ear emergence was used to answer the following questions:

- How does a late S fertilization change storage protein composition?
- Does a late S fertilization have an impact on storage protein composition during the whole grain development?
- If storage proteins are changed by late S fertilization, how does this affect baking quality?
- Are there advantages by applying a late S fertilization in wheat production?

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## Chapter 2

### **Time-dependent Distribution of Sulphur, Sulphate and Glutathione in Wheat Tissues and Grain as Affected by Late Sulphur Fertilization**

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*submitted*

## **2. Time-dependent distribution of sulphur, sulphate and glutathione in wheat tissues and grain as affected by late sulphur fertilization**

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## 2.1. Abstract

Sulphur (S) fertilization has beneficial effects on the yield and protein composition of wheat grain. To understand the impact of S fertilization on storage protein composition, the synthesis of S-containing compounds and their distribution during whole grain development has to be analysed. A pot experiment was conducted with *Triticum aestivum* cultivar Türkis under three S fertilization levels (0 g, 0.1 g and 0.2 g S per pot) and a late S fertilization level (0.1 g S at sowing + 0.1 g S at ear emergence). Stem and leaves, flag leaves, ears and kernels were harvested separately during grain development at ear emergence, milk ripeness and maturity. Sulphate was the major S compound in stem, leaves and ears at the beginning of grain development, with glutathione becoming important later in development for the transport of S into the grain and for the synthesis of S-containing proteins. At milk ripeness, the S content of the plant was sufficient to cover the high sink capacity of grain at all fertilization levels. A discrepancy in S concentration between low and high S fertilization became influential after milk ripeness. The early ratio of N to S in ears at ear emergence reflects the later ratio of N to S in mature grain. Late S fertilization increased sulphate concentrations in the flag leaf within a short time at ear emergence and might prevent S deficiency in wheat grain.

**Key words:** Glutathione, late S fertilization, milk ripeness, sulphate, sulphur, winter wheat.

## 2.2. Introduction

The fertilization of sulphur (S) in adequate amounts and at suitable developmental stages is a prerequisite for the ideal growth and optimal yield of wheat. Likewise, S fertilization is important for the subsequent baking quality of the wheat grain (Haneklaus et al., 1992; Zhao et al., 1999). A former investigation (Zörb et al., 2009) has shown that late S fertilization is suitable for enhancing baking quality in winter wheat. The improved baking quality cannot exclusively be explained by an enhanced nitrogen or sulphur concentration in the flour but rather by specific changes in grain protein composition. In this context, the influence of physiological processes on the synthesis of S-rich storage proteins and important S-rich metabolites such as glutathione in wheat remains uncertain. In addition, the translocation of S-containing compounds such as sulphate and glutathione from vegetative tissues to the grain is a prerequisite for the synthesis of S-rich storage proteins in wheat grain. Therefore, an analysis is necessary of both the composition of grain and the distribution of S-rich compounds in plant tissues such as stem, leaves, the flag leaf, ears and grains under various S fertilization levels.

Glutathione is one major S-containing peptide that can fulfil several functions in plant metabolism. Fitzgerald et al. (1999b) have investigated the impact of S fertilization on the distribution and translocation of sulphate, cysteine and glutathione within the whole wheat plant at various developmental stages. During generative development, glutathione is used as a transport molecule to translocate sulphur from leaves to sink organs (Fitzgerald et al., 1999a). Rennenberg (2001) has further suggested that glutathione acts as a whole-plant signal for sensing S status. Nevertheless, in the last few decades, glutathione has been preferentially discussed with respect to its function as a detoxification reagent for xenobiotics (Schroeder et al., 2007) and heavy metals (Tausz et al., 2004). Furthermore, glutathione in cooperation with ascorbic acid maintains a rigidly regulated redox state within the plant cell (Noctor and Foyer, 1998). Investigations to examine the impact of S fertilization on S distribution within the plant have predominantly been undertaken at low S in comparison with high S fertilization. The impact that variable S fertilization has on S translocation among grain development remains to be established. Furthermore, the adequate timing of S fertilization needs to be examined; for example, does late S fertilization influence the distribution of sulphate and glutathione in various plant tissues? In former studies, investigations into the impact



of S fertilization on the transport, distribution and concentration of S-rich compounds in wheat plants have been undertaken predominantly in hydroponic culture (Anderson and Fitzgerald, 2001; Fitzgerald et al., 2001). In this report, we show the effects of enhanced S fertilization and late S fertilization on the distribution of protein, total S, sulphate and glutathione. These compounds are a prerequisite in S uptake from soil, S assimilation and transport into wheat tissues and mature grain.

### **2.3. Material and Methods**

#### *Plant cultivation*

Experimental conditions with respect to nitrogen and sulphur fertilization, S-poor soil conditions and weather were achieved comparable to those of field trials. Winter wheat cultivar Türkis was grown under the following conditions. Mitscherlich pots were filled with 6 L soil (1/3 loam and 2/3 quartz-sand) and increasing amounts of  $\text{MgSO}_4$ . Three different S fertilization levels were applied with 0 (non-fertilized), 0.1 (low) and 0.2 g (high) S per pot before sowing. Additionally, a late N and S fertilization with 0.1 g S  $\text{pot}^{-1}$  before sowing and 0.1 g S  $\text{pot}^{-1}$  was applied at EC 45 (Lancashire et al., 1991) when the flag leaf sheath opened. Each wheat cultivar was grown in four replicates, respectively. N was applied at three stages (3 x 1 g N,  $\text{NH}_4\text{NO}_3$ ) before sowing, at EC 30 and at EC 45 when the flat leaf sheath opened. The major elements phosphorus (5 g  $\text{CaHPO}_4$   $\text{pot}^{-1}$ ), potassium (3.43 g  $\text{KCl}$   $\text{pot}^{-1}$ ) and magnesium (1 g  $\text{MgCO}_3$   $\text{pot}^{-1}$ ) and the minor elements Cu, Zn and B were applied at optimal concentrations. During plant development, the stem and leaves, flag leaves, ears and kernels were harvested separately at EC 59 (ear emergence), EC 77 (13 days post anthesis, milk ripeness) and EC 89 (maturity) (Tab. 1). Stem and leaves were taken together but the flag leaf was excluded. Ears were fractionated as glumes without kernels. Half of the material was shock-frozen in liquid nitrogen and further ground with a mortar and pestle under liquid nitrogen to achieve a fine powder that was immediately stored at  $-80$  °C. Ground plant material was used for analysis of protein and glutathione concentrations. Half of the material was freeze-dried and further ground to a fine homogeneous powder by using a ball mill. Freeze-dried material was used for the analysis of sulphur and sulphate concentrations.

*Protein and sulphur concentrations*

Protein concentration was determined according to Bradford (Bradford, 1976). Sulphur concentrations were determined according to the Dumas combustion method by using a CNS elemental analyzer (Flash EA 1112 NCS, thermo fisher scientific, Waltham, MA, USA).

*Glutathione concentration*

Glutathione concentration was analysed according to Langenkämper et al. (2006) by using an enzymatic method.

*Sulphate concentration*

Sulphate in freeze-dried samples was extracted by hot water extraction according to Gerendas and Sattelmacher (1997). For extraction, 1.5 mL de-ionized water was added to 0.03 g dried plant material and promptly transferred to a boiling water bath for 5 minutes. After incubation on ice for at least 30 minutes, samples were centrifuged (20 minutes, 18890 g) and proteins were further excluded from the supernatant by extraction with chloroform. Protein-free supernatant was filtered on C18-columns prior to ion-chromatography. If necessary, extracted solutions were diluted prior to analysis by ion-chromatography (ICS 2500, Dionex, Sunnyvale, CA, USA).

*Data, replication, statistics*

All statistical analysis was carried out by using SAS (SAS Institute Inc., Cary, USA, Release 9, 2001). Comparisons of means with respect to the influence of S fertilization and developmental stage were carried out by using the mixed model procedure considering a two-factorial, orthogonal, fully randomized design with a repeated statement for the developmental stage. The homogeneity of variances and of the normal distribution was evaluated by plotting studentized residues over predicted values (means) and by the Shapiro-Wilk test, respectively. Where appropriate, data were log-transformed to maintain homogeneity of variance. In order to maintain an experiment-

wise  $\alpha$  of  $p < 0.05$ , multiple t-tests were adjusted according to Bonferroni-Holm (Horn and Vollandt, 1995). In the following, statistical significance is indicated by small letters for the developmental stage and capital letters for the S fertilization level, respectively. In addition to the biological replicates ( $n=4$ ), two technical replicates of each sample were measured.

## 2.4. Results

### 2.4.1 *Effect of S fertilization on protein concentration*

Sulphur fertilization has no significant impact on protein concentration in kernels (Fig. 1 A). The protein concentration in ears slightly increased with higher S fertilization (Fig. 1 B). The lowest concentrations in ears were observed in non-fertilized wheat plants at milk ripeness. Protein concentration in flag leaves significantly increased with higher S fertilization at milk ripeness (Fig. 1 C). The protein concentration in stem and leaves (excluding the flag leaf) was lower than in flag leaves but increased significantly with S fertilization at the stage of ear emergence and milk ripeness (Fig. 1 D). In stem, leaves and ears, a reduction of protein concentration was observed during grain filling in non-fertilized and low S plants.

### 2.4.2 *Effect of S fertilization on sulphur and sulphate concentrations*

Sulphur fertilization was highly correlated with total S concentration in all plant tissues (ear, flag leaf, stem and leaves) (Fig. 2 B-D). In general, the flag leaf contained the highest S concentrations compared with all other plant tissues. In a comparison of late S fertilization with high S fertilization, no significant differences were found in total S concentrations in all plant tissues at all comparable developmental stages. In stems and leaves (Fig. 2 D) and in flag leaves (Fig. 2 C) and ears (Fig. 2 B), total S concentrations decreased during development with late, high and low S fertilization, except in non-fertilized plants. In kernels, S fertilization had a higher influence on total S concentration at maturity than at milk ripeness (Fig. 2 A). Particularly in the flag leaf

with high and late S fertilization at both ear emergence and milk ripeness (Fig. 2 C), S concentrations were twice as high as in non-fertilized plants.

In kernels, sulphate concentrations decreased during grain development (Fig. 3 A).

In milk-ripe kernels, sulphate concentrations were highly correlated with S fertilization and decreased until maturity. Sulphate concentrations in ears decreased merely with high and late S fertilization during grain development (Fig. 3 B). In all tissues, sulphate concentrations at milk ripeness under high S fertilization were at least twice as high as with low S fertilization. However, the highest sulphate concentrations were detected in ears, flag leaves and stem and leaves with late S fertilization. In flag leaves (Fig. 3 C), non-fertilized and low S wheat plants did not differ in sulphate concentrations. The flag leaf retained extremely high sulphate concentrations until maturity at high and late S fertilization (Fig. 3 C).

#### *2.4.3 Effect of S fertilization on glutathione concentration*

Glutathione concentrations in all plant tissues were highly influenced by S fertilization (Fig. 4). In kernels and ears, the glutathione concentration decreased until maturity (Fig. 4 A, B). The highest glutathione concentrations in comparison with all other plant tissues were detected in milk-ripe kernels (Fig. 4 A). In contrast to ears, the glutathione concentration in flag leaf (Fig. 4 C) and in stem and leaves (Fig. 4 D) remained constant during development. In all tissues, glutathione concentration under high S fertilization was at least twice as high as that with low S fertilization.

#### *2.4.4 Effect of S fertilization on whole plant S, sulphate and glutathione distribution*

Sulphur, sulphate and glutathione concentrations in total in all plant tissues among plant development are illustrated in Fig. 5. Furthermore, large areas illustrate high concentrations and steep courses illustrate increasing concentrations within one tissue. Total S, sulphate and glutathione concentrations (Fig 5 A-C) in whole wheat plants increased with higher S fertilization and showed highest concentrations at milk ripeness. The flag leaf contained the highest concentrations of total sulphur (Fig. 5 A). The only difference between high and late S fertilization was determined for sulphate

concentrations at ear emergence (Fig. 5 B). Kernels contained the highest glutathione concentrations compared with all other tissues (Fig. 5 C).

#### *2.4.5 Effect of S fertilization on N/S ratio*

Sulphur fertilization decreased N/S ratios in all plant tissues (Tab. 2). During grain development, N/S ratios decreased with high and late S fertilization, whereas N/S ratios increased in non-fertilized and low S wheat plants. In kernels, N/S ratios were only slightly influenced by S fertilization. The highest N/S ratios could be observed in stem and leaves at maturity in non-fertilized and low S wheat plants and at milk ripeness in the flag leaf of non-fertilized plants.

## **2.5. Discussion**

Glutathione is extremely important in plant metabolism because of its multiple role, i.e. its function as a redox molecule, as a detoxification molecule for xenobiotics and as the S transport form in various plant tissues and species (Rennenberg, 2001). In former studies, the influence of S fertilization on sulphate and glutathione concentration in wheat has primarily been investigated with regard to the difference between low and high S fertilization (Buchner et al., 2010; Fitzgerald et al., 1999a). Some studies have investigated the impact of S fertilization on the composition of storage protein predominantly within mature wheat grains (Wieser et al., 2004; Zhao et al., 1999; Zörb et al., 2009). Furthermore, the synthesis of S-rich storage proteins in wheat grain proceeds for about 30 days after anthesis (Shewry et al., 2009). Hence, in order to understand the impact of S fertilization on storage protein composition, an examination of S-dependent compounds in all plant tissues during whole grain development is of primary importance. However, our work closes a gap in the lack of knowledge concerning the distribution of S-containing compounds after non-fertilization, after low and high S fertilization and after a late S application during whole grain development.

### 2.5.1 *Impact of S fertilization on total S, sulphate and glutathione concentration*

Wheat has the ability to compensate for a decreased synthesis of S-rich proteins at S deficiency by the synthesis of S-poor proteins (Wieser et al., 2004). This aspect was also detected on a 'whole plant' level (Fig. 1). Although, a clear correlation has been observed only in flag leaves at milk ripeness between protein concentration and S fertilization (Fig. 1 C), a high correlation of S fertilization with total S, sulphate and glutathione concentrations could be detected in all tissues (Fig. 2 and Figs. 3, 4, 5). Especially in flag leaves, total S concentrations correlated positively with S fertilization (Fig. 2 C). Furthermore, the total S concentration in flag leaves was twice as high as that in stem and leaves (Fig. 2 C and D). Thus, the flag leaf represents a strong source for supplying the developing grain with sulphur. Of further interest, S fertilization has only a slight influence on total S concentration in kernels at milk ripeness (Fig. 2 A). In contrast, glutathione and sulphate are highly influenced by an increasing S fertilization (Fig. 2 A; Fig. 3 A). Therefore, S-rich proteins or other S-rich metabolites might contribute to total S concentration of the plant. Bourgis et al. (1999) have found S-methylmethionine to be an important metabolite that is transported in high amounts towards the grain via the phloem.

Sulphate is the S-containing anion that is absorbed by roots, transported to the shoot, assimilated to the S-containing amino acid cysteine and later converted to the tripeptide glutathione. By applying late S fertilization, sulphate concentrations in flag leaves at ear emergence were extraordinarily higher compared with that under high S fertilization (Fig. 3 C). However, at this developmental stage, no difference was seen in glutathione concentration on comparing high and late S fertilization (Fig. 4 C). Furthermore, in kernels, the sulphate concentration did not differ between high and late S fertilization (Fig. 3 A). As a result, sulphate appears to be primarily transported to the flag leaf and not to the grain (Fig. 5 B). Moreover, late S fertilization is suitable in order to supplement the developed sulphate deficit in flag leaf after low S fertilization within a short time period at the beginning of grain development (Fig. 3 C; Fig. 5 B).

From another aspect, Fitzgerald et al. (2001) have described higher glutathione concentrations in comparison with sulphate in developing grains in plants fertilized with high S amounts. Our data confirm this aspect (Fig. 3 and 4 A). Especially at milk ripeness, glutathione concentrations were 10-fold higher than sulphate concentrations in

milk-ripe kernels. At the whole plant level (Fig. 5 C), glutathione concentrations increased predominantly in kernels at milk ripeness and represented the highest proportion of S-rich components investigated. Kernels might instead require S for the synthesis of storage proteins by utilization of glutathione than S from the *de novo* synthesis of cysteine from absorbed sulphate. Of interest is the way in which sulphate and glutathione concentrations are influenced by the various amount and the timing of S fertilization. Fitzgerald et al. (1999b) have shown that low-S plants preferentially use S from the protein breakdown in leaves to transfer S to the grain. This aspect is confirmed by our findings of decreasing protein concentrations in leaves during grain development (Fig. 1 B-D).

The high discrepancy of sulphate concentrations on comparing low and high S fertilization in all plant tissues is also of interest (Fig. 3; Fig. 5 B). Sulphate concentrations in low S plants remain low during grain development, whereas a decrease of sulphate concentrations occurs in stem and leaves, ear and kernels at high and late S fertilization until grain maturity (Fig. 5 B). At low S fertilization, sulphate is directly used for the assimilation of cysteine, whereas at high S fertilization, sulphate uptake is too high for the S to be directly assimilated. Thus, surplus sulphate is stored in vacuoles of leaf cells until assimilation. This aspect can be explained by the highly regulated feedback mechanisms that take place during sulphate assimilation and glutathione synthesis (Droux, 2003; Hell et al., 1995; Höfgen et al., 2001; Kopriva and Rennenberg, 2004; Yi et al., 2010). Our data further show that sulphate accumulates at ear emergence with high and late S fertilization (Fig. 3 B-D), whereas glutathione concentrations do not increase concomitantly (Fig. 4 B-D). As a result, glutathione alone does not repress sulphate uptake. To date, the internal signal that might mediate the S status from the shoot to the roots remains unknown (Anderson and McMahon, 2001). Glutathione alone might not be the transducing signal for the feedback mechanism to the roots.

When comparing high with late S fertilization at both milk ripeness and maturity, late S fertilization has no adverse effects on protein, sulphur, sulphate or glutathione concentration in any tissue at any time (Fig. 5). Late S fertilization is a procedure that can help to prevent S deficiency (Zörb et al., 2010), regardless of whether S fertilization is applied by a single dressing at sowing or by a separated dressing. Our data reveal that a latent S deficiency at ear emergence can be compensated by late S dressing, as wheat

plants can replenish sulphate deficits within a short time. Late S fertilization is further thought to enhance baking quality by increasing HMW (high molecular weight) glutenin concentration (Zörb et al., 2009).

### 2.5.2 *Tissue specific response to S fertilization*

The highest glutathione concentrations at the whole plant level are observed in milk-ripe kernels (Fig. 4 A). The N/S ratios in milk-ripe kernels are only slightly different (Tab. 2). This aspect underlines the high sink capacity of kernels at the time of highest protein synthesis. Furthermore, the grain can express its own sulphate transporters (Buchner et al., 2010). The grain is able to attain sulphur by both the assimilation of sulphate and the uptake of glutathione from vegetative tissues as follows. Further, the wheat grain has its own enzymatic equipment to catalyze the S assimilation of sulphate to cysteine (Fitzgerald et al., 2001) and to degrade glutathione to cysteine.

Ears may only play a role as a source tissue for kernels comparable with pods in soybean. Ears lose protein (Fig. 1 B) and sulphur, except in non-fertilized plants (Fig. 2 B), and further lose sulphate only with high S fertilization (Fig. 3 B). Sunarpi and Anderson (1997) have examined the allocation of S in generative growth of soybean in which sulphate is assimilated to S-containing amino acids and glutathione within pods. Later during development, seeds acquire S from pods presumably in the form of glutathione, which is degraded to cysteine for protein synthesis.

In flag leaves, the expression of high-affinity sulphate transporters increases after anthesis in sulphur-starved plants (Buchner et al., 2010). During this developmental stage, large amounts of S are lost from the flag leaf (Fig. 2 C; Fig. 5 A) in order to supply the developing kernels. The flag leaf serves as both a source and a sink organ. For stem and leaves, the flag leaf is a sink organ, as sulphate and glutathione concentrations do not decrease during grain development. However, the flag leaf has a high impact of source capacity because large amounts of S are transported to the grain. Buchner et al. (2010) have shown the increasing expression of sulphate transporters underlining the high demand for S in leaves during grain development. The stem and leaves play a minor role in directly supplying the grain with sulphur; they mobilize protein independently from S fertilization presumably via senescence (Howarth et al.,



2008) and transfer sulphur only under high and late S fertilization, whereas glutathione concentrations remain constant. In stem and leaves, sulphate might maintain its role as the transport form of S through development (Buchner et al., 2010), whereas the main transport form of S to generative tissues is glutathione. Buchner et al. (2010) have further revealed that the impact of S transport to the grain increases during development in older leaves as the expression of sulphate transporters increases. Stem and leaves might primarily need glutathione for maintaining the redox state but, later in development, glutathione largely contributes to the supply of S-containing compounds to the grain.

During development, N/S ratios decrease under high and late S fertilization but increase in non-fertilized and low S plants (Tab. 2). This aspect indicates the high capacity for the mobilization of S in high S plants. Furthermore, the N/S ratios in ears early at ear emergence and milk ripeness reflect the N/S ratios of mature wheat kernels. Thus, N/S ratios at ear emergence can be used as indicators for the S concentration of kernels at maturity.

In future, other S-containing metabolites that are synthesized during S assimilation and glutathione synthesis need to be examined to investigate their impact on S translocation from vegetative to generative tissues. We need to establish whether the higher concentrations of S-containing compounds in kernels are responsible for better protein quality by increasing the S content of storage proteins or whether other factors are involved in quality improvement.

## 2.6. Conclusions

Sulphate is the major S compound in stem, leaves and ears at the beginning of grain development. Glutathione becomes an important transport form for S during the synthesis of storage protein in the grain. Kernels mobilize S for the synthesis of storage proteins from the degradation of glutathione rather than from the *de novo* synthesis of cysteine from absorbed sulphate. At milk ripeness, the whole plant S content is sufficient to cover the sink capacity of kernels. Discrepancy in S concentrations between low and high S fertilization become influential after milk ripeness. The early ratio of N to S in ears at ear emergence reflects the later ratio of N to S in mature grain. Late S fertilization increases sulphate concentrations in the flag leaf within a short time at ear emergence and can prevent S deficiency.

### **Acknowledgements**

We appreciate the excellent technical assistance of Stephanie thor Straten and Martina Bach.

Figures

Fig. 1

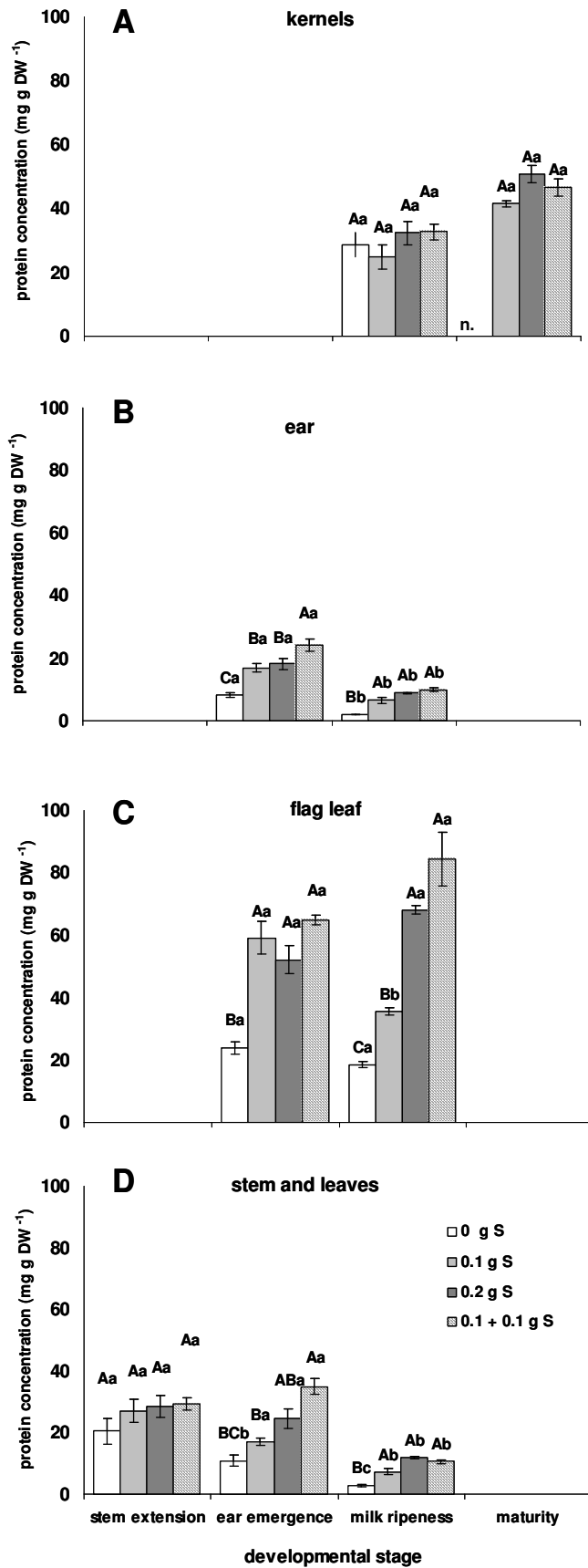


Fig. 2

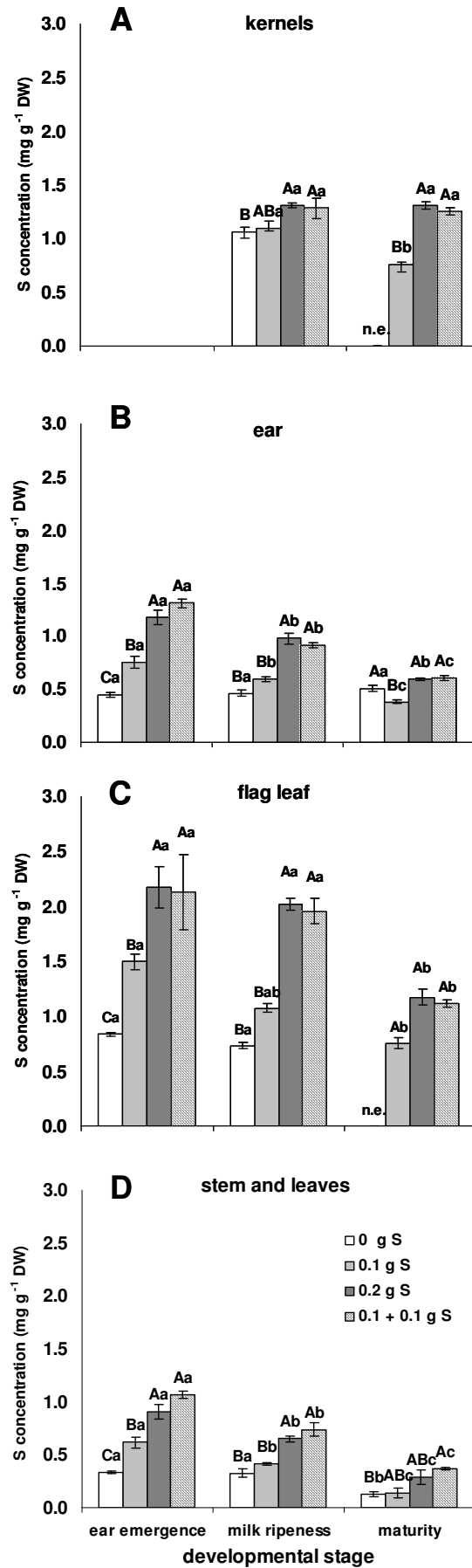


Fig. 3

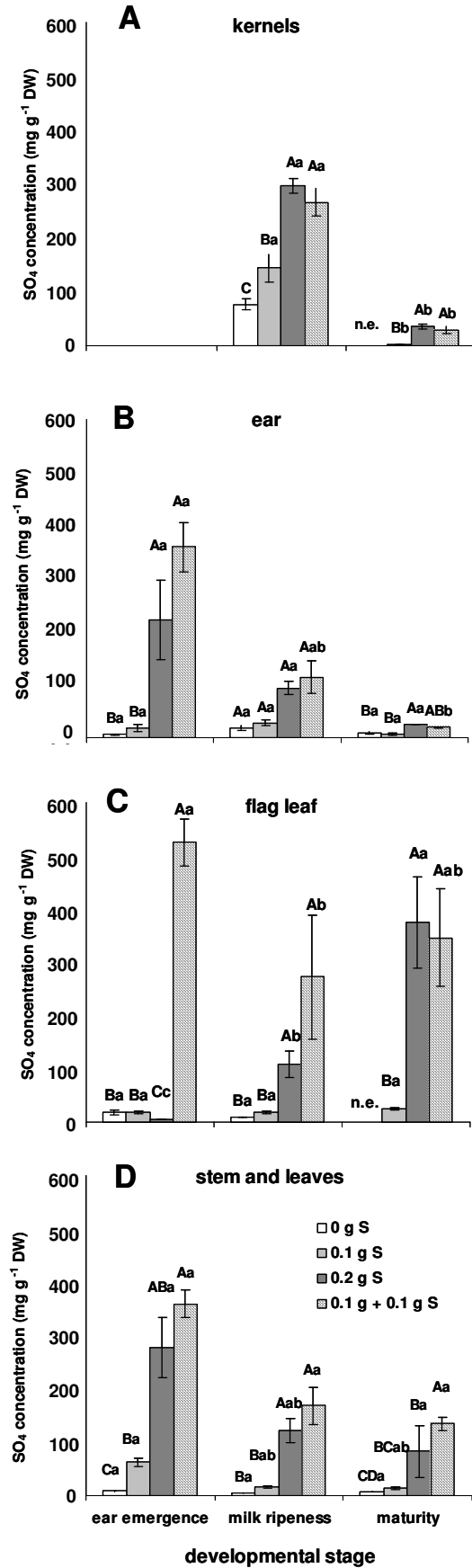


Fig. 4

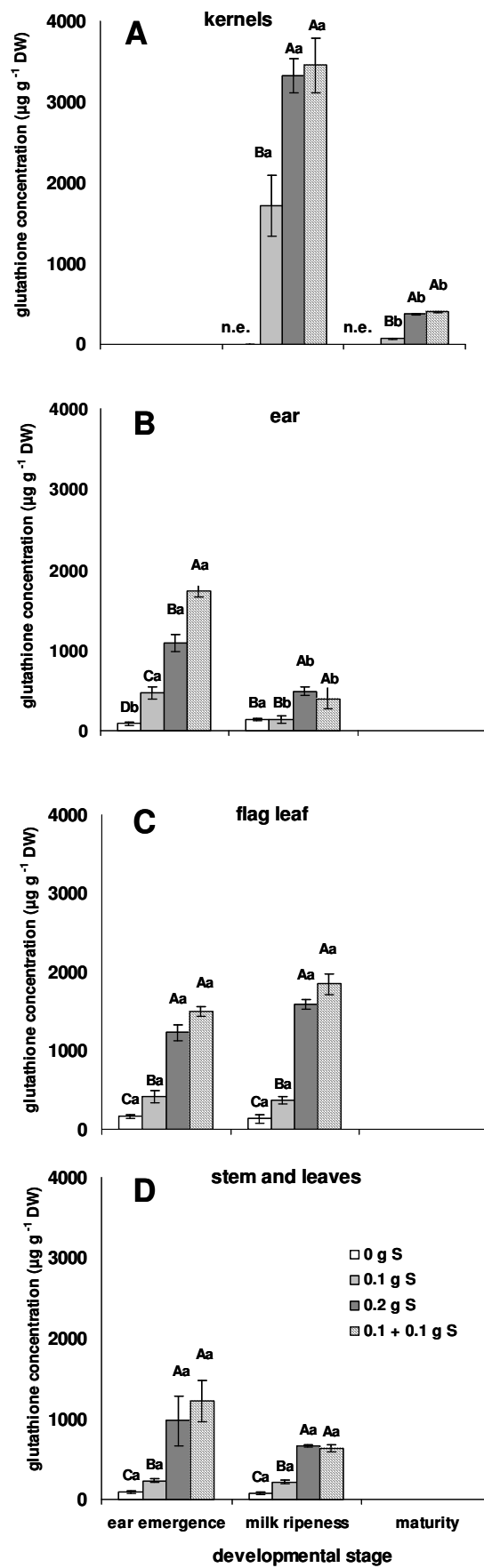
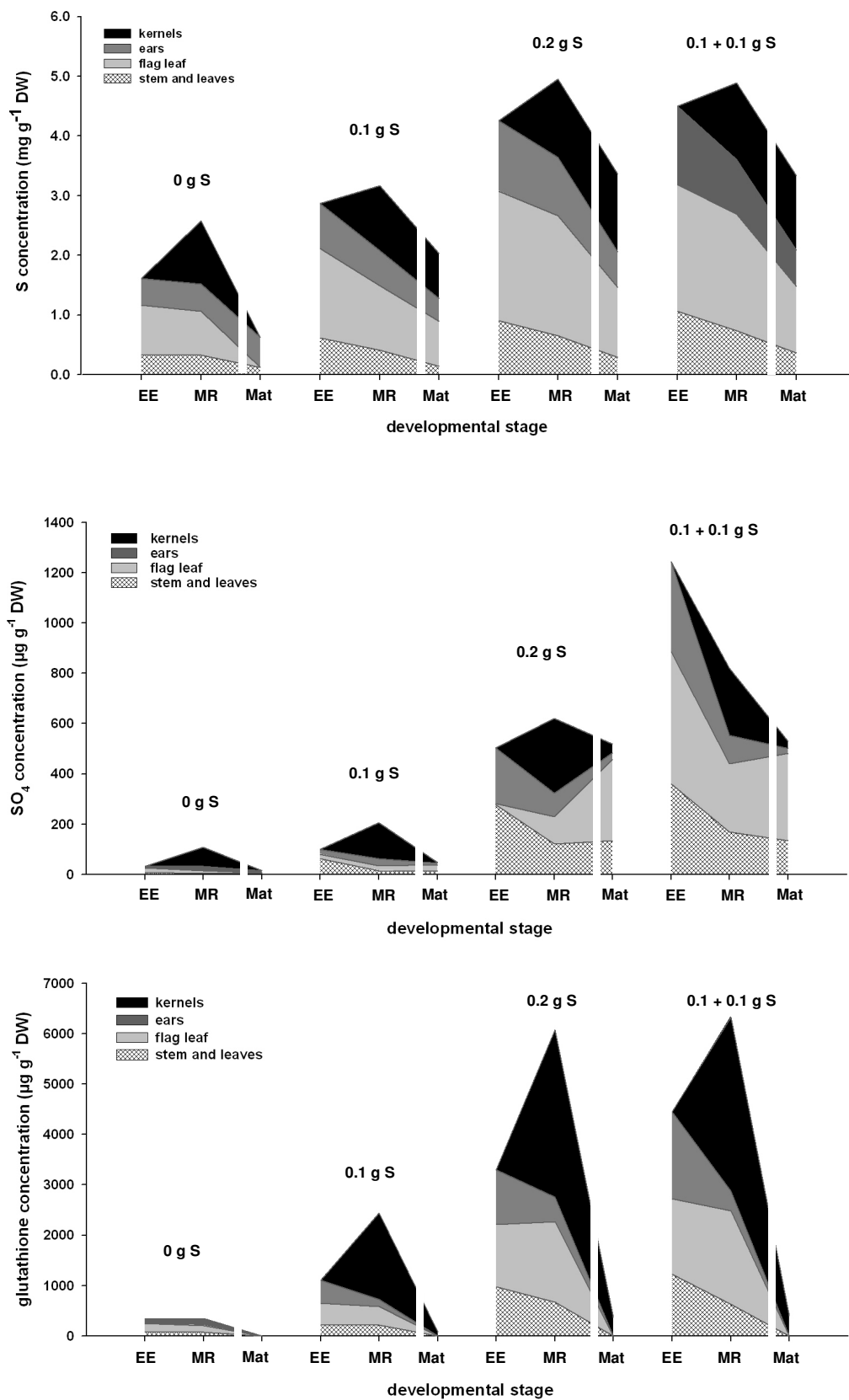


Fig. 5



## Tables

Tab. 1

Developmental Stage (EC)	Days post anthesis (dpa)	Harvesting Date	Plant tissues examined
Ear emergence (59)	-	May 20 <sup>th</sup> 2009	Stem and leaves Flag leaf Ear
Milk ripeness (77)	13	June 9 <sup>th</sup> 2009	Stem and leaves Flag leaf Ear Kernels
Maturity (89)	55	July 21 <sup>st</sup> 2009	Stem and leaves Flag leaf Ear Kernels

Tab. 2

<b>Impact of S fertilization on N/S ratios in wheat tissues</b>			
<i>kernels</i>	<b>ear emergence</b>	<b>milk ripeness</b>	<b>maturity</b>
0 g S	.	22	n.e
0.1 g S	.	22	28
0.2 g S	.	18	16
0.1 + 0.1 g S	.	18	16
<i>ears</i>	<b>ear emergence</b>	<b>milk ripeness</b>	<b>maturity</b>
0 g S	41	41	49
0.1 g S	26	27	25
0.2 g S	16	17	13
0.1 + 0.1 g S	16	17	14
<i>flag leaf</i>	<b>ear emergence</b>	<b>milk ripeness</b>	<b>maturity</b>
0 g S	42	63	n.e.
0.1 g S	21	24	6
0.2 g S	16	16	4
0.1 + 0.1 g S	16	15	4
<i>stem and leaves</i>	<b>ear emergence</b>	<b>milk ripeness</b>	<b>maturity</b>
0 g S	41	41	161
0.1 g S	20	12	33
0.2 g S	14	11	7
0.1 + 0.1 g S	13	9	5



## Figure captions

Fig. 1: Impact of sulphur fertilization on elemental S concentration within **A** kernels, **B** ear, **C** flag leaf and **D** stem and leaves. At stem extension and ear emergence, kernels had not yet developed. At stem extension, ears and flag leaves had not yet developed. Protein concentration at maturity in ears, flag leaves, stem and leaves could not be examined because the material was too dry. Error bars represent  $\pm$  standard error of four independent pot replications. Statistical significance ( $p \leq 0.05$ ) is indicated by small letters for the developmental stage and capital letters for the S fertilization level.

Fig 2: Impact of sulphur fertilization on sulphate concentration within **A** kernels, **B** ear, **C** flag leaf and **D** stem and leaves. At ear emergence, kernels had not yet developed. Error bars represent  $\pm$  standard error of four independent pot replications. Statistical significance ( $p \leq 0.05$ ) is indicated by small letters for the developmental stage and capital letters for the S fertilization level.

Fig. 3: Impact of sulphur fertilization on glutathione concentration within **A** kernels, **B** ear, **C** flag leaf and **D** stem and leaves. At ear emergence, kernels had not yet developed. Glutathione concentration at maturity in ears, flag leaves, stem and leaves could not be examined because the material was too dry. Error bars represent  $\pm$  standard error of four independent pot replications. Statistical significance ( $p \leq 0.05$ ) is indicated by small letters for the developmental stage and capital letters for the S fertilization level.

Fig. 4: Impact of sulphur fertilization on protein concentration according to Bradford (1976) in **A** kernels, **B** ear, **C** flag leaf and **D** stem and leaves. Error bars represent  $\pm$  standard error of four independent pot replications. Statistical significance ( $p \leq 0.05$ ) is indicated by small letters for the developmental stage and capital letters for the S fertilization level.

Fig. 5: Impact of sulphur fertilization on total **A** elemental S, **B** sulphate and **C** glutathione concentration in wheat plant among the various developmental stages: EE = ear emergence; MR = milk ripeness and Mat = maturity. At ear emergence, kernels had not yet developed. Glutathione concentration could not be determined in mature stem and leaves or in flag leaf and ears. No kernel yield occurred at the 0 g S fertilization level.

Tab. 1: Concepts for harvesting plant tissues. Late N ( $\text{NH}_4\text{NO}_3$ ) and S ( $\text{MgSO}_4$ ) were applied on May 30<sup>th</sup> (EC 45).

Tab. 2: Impact of S fertilization on N/S ratios in stem and leaves and in flag leaf, ear and kernels during grain development. At ear emergence, kernels had not yet developed. Flag leaf and kernels at 0 g S fertilization could not be harvested.

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## Chapter 3

### **Quantitative Protein Composition and Baking Quality of Winter Wheat as Affected by Late Sulfur Fertilization**

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## Chapter 4

### **Impact of Nitrogen and Sulfur Fertilization on Gluten Composition, and Baking Quality of Wheat**

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**4. Impact of nitrogen and sulfur fertilization on gluten, composition and baking quality of wheat**

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## 4.1 Abstract

Wheat has a broad genetic potential for the expression of various gluten proteins and is strongly influenced by the environment, e. g. with respect to the fertilization of nitrogen (N) and sulfur (S) during wheat plant development. Nitrogen and S availability markedly change wheat flour and baking quality. However, without adequate management of N and S fertilization, the genetic potential of wheat cannot be exploited. Nitrogen fertilization primarily affects the concentration of gliadines and glutenines as it is source-regulated. Storage protein concentration, particularly of gliadine and glutenine, is highly correlated with loaf volume. The use of three instead of one dressing of fertilizer, especially as a late dressing at ear emergence, the gliadine and glutenine concentration of the grain and, furthermore, loaf volume can be enhanced. However, not only quantity, but also the quality of gluten proteins is important for their composition and characteristics. Despite protein amount being only slightly influenced by increasing S fertilization levels, the composition of gluten proteins is highly affected. Increasing S availability for plants enhances S-containing amino acids, namely cysteine and methionine, which are foremost in S-rich gluten proteins. For an improvement of baking quality, a high concentration of S-rich proteins is of particular importance in order to form an appropriate gluten network within the dough. Additionally, a late S dressing can further improve baking quality, since such a dressing increases the synthesis of high molecular weight (HMW) glutenins that considerably affect baking quality in wheat.

S-rich components such as sulfate and glutathione are, moreover, important molecules for the transport and supply of S to the grain. Both these molecules represent important S transport forms from source to sink organs. The potential to synthesize large amounts of storage protein depends on the ability of the plant to take up and to transport S into sink organs such as ears and kernels. Sulfur and glutathione are therefore important molecules functioning as actuators during plant growth and grain development. Furthermore, glutathione can function as internal plant signal for the S status of the plant. In terms of baking quality, glutathione is able to interact with gluten proteins responding in a change of existing disulfide bonds between gluten proteins and resulting in a different rheological property and baking quality. Concerning the interaction of N and S in wheat, a well-balanced N/S ratio is exceedingly important for suitable gluten

and baking quality. If high N fertilization is accompanied by inadequate S fertilization, S deficiency is provoked, resulting in changed gluten protein composition and a loss of nutritional quality of the grains.

## 4.2 Introduction

Gluten proteins in cereals belong to the family of plant storage proteins and primarily provide nitrogen (N) and sulfur (S) stores for the growth of seedlings. In terms of human and animal nutrition, these storage proteins are not only a source of essential amino acids, but also of particular importance for product quality. Especially in wheat, gluten proteins are highly important, since they have special characteristics, appearing exclusively in wheat, for dough preparation and baking quality (Zhao et al., 1999b).

Fertilization of wheat plants with N and especially with S has been extensively discussed, since environmental S depositions from the atmosphere have decreased during the last decades (Vestreng et al., 2007). Sulfur dioxide (SO<sub>2</sub>) from the atmosphere thus no longer acts as a sufficient source for S. Hence, S application to the soil in any form and its management during plant development needs to be further investigated. Although wheat is not such a high-S-demanding crop plant as canola, S deficiency becomes obvious as soon as S fertilization is inadequate. Sulfur deficiency in wheat results in a change of color from dark green to light-green in young leaves (Bell et al., 1995), whereas N deficiency is diagnosed by the lightening of old leaves. Consequently, an inadequate nutrient supply results in reduced yield (Zhao et al., 1999a), the lower production of S-containing amino acids and S-rich storage proteins (Wieser et al., 2004), and low baking quality. These aspects, when taken together, lead to lower profits for farmers and the baking industry.

Furthermore, as a reinforcing situation, wheat breeding during the last few decades has focused mainly on increasing yield and resistance against plant pathogens and only to a lesser extent on baking quality. As a consequence, yield has increased at the expense of protein concentration in grain (Feil, 1997; Statistisches Bundesamt, 2010).

Each wheat cultivar has its characteristic, genetically determined pattern of gluten proteins, which have a major influence on baking quality. Irrespective of the genetic

background, N and S fertilization further highly influence the composition of storage proteins. Thus, fertilization is a means to influence not only grain yield, but also grain quality. As early as the middle of the last century, Finney and Barmore (1948) showed that wheat cultivars exhibit different slopes with respect to loaf volume, and thus baking quality increases with protein concentration independently of the genetic background of the cultivars. Is it therefore possible to increase both yield and baking quality by adequate N and S fertilizer application?

The main focus of this chapter is to demonstrate the impact of N and S fertilization on the modification of gluten proteins and their rheological characteristics and baking quality parameters. Nitrogen and S fertilization can be managed in several ways: (1) the amount of N and S fertilizer applied, (2) the application of soil or foliar fertilizers, and (3) the application at different developmental stages of plant growth. Moreover, an elucidation of the way that N and S interact during the formation of gluten proteins is of interest.

### **4.3 Gluten protein composition and synthesis**

Since T. J. Osborne's (1924) publication concerning the extractability of storage proteins, they have been classified into four groups in wheat: the albumins and globulins, which are mainly metabolic and structural proteins (Goesaert et al., 2005), are extractable with water and NaCl solution, respectively. Other storage proteins such as the prolamin or gluten proteins are divided into gliadins, which are extractable with ethanol, and glutenins, which need to be extracted with reducing agents because of covalent cross-links such as disulfide bonds (Osborne, 1924; Shewry, 1995). Gliadins and glutenins account for about 75 % of mature wheat grain proteins (Shewry and Halford, 2002) and are the most important factors for the remarkable breadmaking properties of wheat.

Since the role of globulins in baking quality is still not fully understood (Shewry and Halford, 2002), this chapter will focus on gliadin and glutenin proteins. Gluten proteins further consist of subunits that contain no (S-poor), low (high molecular weight glutenin subunits, HMW-GS), or high (S-rich) S amounts in the form of cysteine (Shewry and

Halford, 2002). Gliadin proteins are monomeric structures that can only form intramolecular subunits, whereas glutenins are able to form both intra- and inter-molecular subunits. B- and C-type low molecular weight (LMW) subunits of glutenin, and  $\gamma$ - and  $\alpha$ -gliadins are S-rich molecules and contain about 2-3% cysteine. D-type LMW subunits of glutenins and  $\omega$ -gliadins are S-poor and contain only 0 to <0.5% cysteine. Cysteine possesses a free thiol-group that is able to form disulfid cross-links with other cysteine-containing molecules. HMW glutenins contain repetitive domains that may be intrinsically elastic because of  $\beta$ -turns in the protein structure that are bordered by  $\alpha$ -helices on the N- and C-terminal domain of the molecule. Additionally, HMW-GS are notably divided into two types according to their genetic background: y-type HMW-GS that are of low molecular weight and x-type HMW-GS subunits that are of high molecular weight. The y-type HMW-GS contain more SH-groups at the N-terminal domain than the x-type HMW-GS (Shewry et al., 1992). Further information concerning glutenin composition can be obtained by reference to the above-mentioned reviews on HMW glutenin subunits by Shewry et al., (1992) and Shewry and Halford (2002).

Gluten protein expression is highly tissue-specific and does exclusively occur in seeds during grain development. The expression of gluten proteins is further regulated by available N and S metabolites that are synthesized during plant growth (Tabe et al., 2002). Nitrogen is on first instance important for amino acid and protein synthesis. Therefore, the next chapter will discuss the role of N fertilization on gluten composition and its effect on baking quality.

## **4.4 The role of nitrogen fertilization in gluten formation and the resulting baking quality**

### *4.4.1 Assimilation and translocation of nitrogen within the wheat plant*

In wheat plant roots, nitrate transporters are mainly responsible for the uptake of nitrate ( $\text{NO}_3$ ) into the root symplast (Yin et al., 2007). Nitrogen is also taken up by wheat roots in the form of ammonium ( $\text{NH}_4^+$ ) (Cox and Reisenauer, 1973). Ammonium, however, cannot be stored in vacuoles in the same manner as nitrate, and furthermore,

ammonia ( $\text{NH}_3$ ) is a cell-toxic compound that needs to be converted into amino acids or amides as rapidly as possible. Most plant species prefer to take up N in the form of nitrate, although some species do indeed take up N in the form of ammonium. This can further be observed under special soil conditions, such as low soil pH and low redox potential (Ismunadji and Dijkshoorn, 1971). For an adequate growth rate and yield of wheat, both forms of N (nitrate and ammonium) have to be applied, since the application of only one N form has adverse effects on the uptake of other nutrients and further on pH regulation (van Beusichem et al., 1988).

In brief, N is assimilated as follows.  $\text{NO}_3$  is taken up into the cytosol of root cells and further converted to nitrite by nitrate reductase. In the next step, nitrite is transported to chloroplasts or plastids and is converted to  $\text{NH}_4$  by nitrite reductase.  $\text{NH}_4$  is the cation that is used to form the first amino acids in plant metabolism: glutamine and glutamate. In the plastids (roots) and chloroplasts (leaves), glutamine synthetase and glutamate synthase (GOGAT) convert  $\text{NH}_4$  into glutamine and glutamate (Mifflin and Habash, 2002). These amino acids are now available to synthesize all the other amino acids and to build up metabolic and structural proteins that are needed during the vegetative growth of wheat plants. Nitrogen assimilation takes place in both root and leaf cells.  $\text{NO}_3$  is transported from roots to leaves via the xylem. If  $\text{NO}_3$  is present to excess in leaves, it is stored in vacuoles and can be regenerated as soon as  $\text{NO}_3$  concentrations decrease in the cytosol. During generative growth,  $\text{NO}_3$  concentrations in the soil without additional fertilization is not sufficiently supplied, and the high demands of sink tissues cannot be met. Therefore, plant organs, especially the flag leaf, regenerate N from protein degradation to meet the N demands of the developing grain (Gooding et al., 2007). Mainly glutamine and asparagine are transported to the grains via the phloem to synthesize storage proteins (Macnicol, 1977; Anderson and Fitzgerald, 2001). The wheat grain has different potentialities for attaining N. First, as discussed above, N that has been stored during vegetative growth can be mobilized, or second, further N can be taken up directly from the soil (deRuiter and Brooking, 1996). If N is not limiting, the primary source is N from vegetative tissues (60-92%) that has previously been assimilated (Simpson et al., 1983). Furthermore, Martre et al. (2003) have found N in grain to be source-regulated, i. e. total grain N is regulated by N in vegetative tissues as incorporated prior to anthesis (Barneix and Guitman, 1993; Fuertes-Mendizábal et al.,

2010) but not by the activity of the grain namely the sink tissue. However, increasing N fertilization enhances the N concentration in the leaves of wheat and further provides the potential to obtain higher amounts of N for translocation to the grain and therefore the synthesis of gluten proteins.

#### *4.4.2 Nitrogen fertilization increases the amount of gluten proteins. Is this all we know?*

Nitrogen fertilization increases the protein concentration in all wheat plant tissues, and especially in wheat grain (Finney and Barmore, 1948). First and foremost, an increase in N fertilization enhances grain yield. However, Gauer et al. (1992) and Garrido-Lestache et al. (2005) have observed stagnating grain yield after N rates of 120 kg ha<sup>-1</sup> and 100 kg ha<sup>-1</sup>, respectively, despite protein concentration still increasing. The enhancement of grain protein concentration by incremental additions of N fertilizer is limited in the same way (Barneix et al., 1992; Triboi and Triboi-Blondel, 2002). After reaching a maximum N concentration in grain, only the N concentrations within the vegetative plant parts increase on additional N application. Mechanisms mediated by ammonium and glutamine reduce the expression of nitrate transporters in roots by negative feedback reactions. As long as storage protein synthesis is in progress, ammonium and glutamine concentrations will remain low and result in a constant N uptake (Foulkes et al., 2009). Furthermore, application at different stages of plant development influence grain yield and N concentration. Early N fertilization during vegetative growth enhances grain yield by influencing kernels per ear and ears per plant. Nitrogen fertilization at anthesis predominantly benefits grain N and therefore grain protein concentration (Jahn-Deesbach and Weipert, 1964; Wuest and Cassman, 1992).

Investigations of gluten protein fractions have shown that Osborne fractions are differently affected by N fertilization. Unlike gliadin and glutenin proteins, albumins and globulins are hardly influenced (Wieser and Seilmeier, 1998; Fuertes-Mendizábal et al., 2010). Wieser and Seilmeier (1998) have provided further insight into the gluten protein composition of various wheat cultivars that were raised on different N regimens.

Interestingly, the quantity of gliadins increases more than the quantity of glutenins. These findings have been confirmed by Triboi et al. (2000) in field experiments.

With respect to the pattern of gliadin and glutenin subunits, the quantity of S-poor gluten proteins such as  $\omega$ -gliadins and HMW-glutenins is mainly enhanced after increasing N fertilization. Wieser and Seilmeier (1998) have further investigated the HMW subunits, and observed that x-type glutenins are more influenced by N fertilization than y-type glutenins. X-type glutenins contain fewer thiol-groups than do y-type glutenins (Shewry et al., 1992). As a consequence, N fertilization enhances primarily S-poor subunits by increasing the expression of S-poor gluten subunit genes. Prieto et al. (1992) have observed increasing  $\omega$ -gliadin concentrations with increasing N fertilization in agreement with the findings by Wieser and Seilmeier (1998).

Recent investigations concerning late N fertilization have provided new insights concerning gluten protein modification. Late fertilization has the following advantages: late N fertilization at ear emergence enhances protein concentration and, furthermore, recovers deficiency symptoms, since fast nutrient translocation mechanisms exist in plants. For example, Foulkes et al. (2009) have observed that N deficiency symptoms at anthesis can be even recovered by a later N fertilization. Tea et al. (2007) have examined the translocation of N and S after a late foliar application and found high recovery rates in leaves and grain with a beneficial impact on N/S ratios and protein concentration. Therefore, under inconvenient weather conditions with high or no precipitation, a split application of nutrients prevents leaching or helps to enable nutrient uptake by foliar application, respectively. Furthermore, the application of N at a time point when demand for N is high makes sense. This high demand occurs predominantly during the first few days of grain development, when proteins have started to be synthesized (Emes et al., 2003; Shewry et al., 2009).

Unlike increasing N fertilization treatments that predominantly increase gliadin concentration, a late application of N, when the flag leaf is just visible, mainly influences the concentration of glutenins (Fuertes-Mendizábal et al., 2010). With respect to the subunits of gluten proteins, each HMW-GS evidently increases with increasing N fertilization. Although HMW glutenins are minor components of gluten

proteins, compromising approximately 12% of all gluten proteins, these glutenin subunits notably account for 45-70% of variation in baking quality (Shewry et al., 2000). This aspect largely explains the variation in baking quality after different N fertilization treatments.

#### *4.4.3 Nitrogen fertilization increases baking quality, but the question is how?*

As early as 60 years ago, Finney and Barmore (1948) showed convincingly that the protein concentration in wheat flour of individual cultivars is highly correlated with loaf volume. However, the parameter that is responsible for this high correlation remains unknown. Parameters such as the gliadin/glutenin ratio (MacRitchie, 1980; Reinbold et al., 2008), amino acid composition (Granvogl et al., 2007), and thiol and disulfide status (Graveland et al., 1978) have been considered as possible explanations for the correlation but have failed to account for the correlation completely. The interdependency seems to be more complex than can be explained by only one parameter. However, can single gluten subunits indeed explain the impact on baking quality?

Many direct and indirect methods are available to examine baking quality on dough or on gluten proteins. The standardized baking test, for example the rapid-mix-test (RMT) (Pelshenke et al., 1978) is a direct method for examining loaf volume, dough consistency during preparation, and sensory parameters. Other methods such as sedimentation value, extensigram, alveogram, and mixograph-test are only capable of predicting loaf volume by the correlation of the flocculation of gliadins and glutenins, elasticity, resistance, and mixing properties, respectively, with loaf volume. Despite the standardized baking test being the only direct method for obtaining information about loaf volume, particularly in small-scale investigations such as pot experiments, indirect methods are appropriate for providing information about the impact of single gluten proteins on rheological parameters.

Several investigations have examined both the impact of N fertilization on baking quality parameters and the change of individual gluten proteins (Luo et al., 2000; Flæte



et al., 2005; Fuertes-Mendizábal et al., 2010). Flæte et al. (2005) have observed increasing sedimentation values and resistance of dough ( $R_{\max}$ ) in gluten extensigraph experiments when comparing high N with low N fertilization levels. A diminished S concentration in gluten proteins as a result of inadequate S fertilization has been assumed to explain the increased resistance of dough. To underline the obvious impact of S, Popineau et al. (1994) used balanced N and S levels and concluded that this combination did not result in increased  $R_{\max}$ .

Furthermore, two-dimensional (2D)-gel electrophoresis has been used to explain the effect of increased N fertilization on protein composition. Flæte et al. (2005) have found two proteins to be changed by increased N fertilization. However, these proteins have been identified to be non-gluten proteins such as glyceraldehyde-3-phosphate dehydrogenase and one serpin protein. With respect to the sedimentation value, Luo et al. (2000) have also compared early N fertilization at booting with late N fertilization at flowering and have observed the same positive impact of N fertilization on the sedimentation value as that reported by Flæte et al. (2005), irrespective of whether either early or late N application was conducted. Unlike Flæte et al. (2005), who used extensibility tests on dough to elucidate the impact of single gluten proteins on baking properties, Luo et al. (2000) used a mixograph test. The mixograph test revealed that the mid-line peak value, but not the mid-line peak time, was enhanced with N fertilization. To conclude, both groups of authors found an increase of dough resistance and a decrease of extensibility attributable to N fertilization. These quality parameters indicate reduced baking quality, since higher resistance and lower extensibility account for a dough that is firm and therefore proves less well on dough fermentation.

Are there any hints in gluten composition that can explain the increased resistance? Ayoub et al. (1994), for example, have investigated both N rate and timing on loaf volume and found that an increasing N rate and late N fertilization increases both, the protein concentration and the loaf volume. Evidently, increasing N fertilization produces wheat doughs with lower extensibility and higher resistance, since those gluten proteins, which are poor in S, increase. Fuertes-Mendizábal et al. (2010) have further elucidated the effect of a late N application on baking properties and additionally found explanations in the gluten composition. Baking quality parameters examined on

an alveograph increase with both an increasing N rate and a late N application. Dough strength increases by 50% with increasing N rate, whereas splitting enhances dough strength by about 80%. A late N application obviously has pivotal effects on both total protein concentration and baking quality characteristics. Investigations into the effects of late N fertilization on gluten composition by Fuertes-Mendizábal et al. (2010) have further enlightened the impact of individual proteins on dough characteristics. The x-type HMW-GS, which contain a smaller amount of SH-groups, are more affected by N fertilization than are y-type HMW-GS. Furthermore, late N fertilization seems to enhance all individual HMW-GS and thus increases dough strength and extensibility (Fuertes-Mendizábal et al., 2010). However, an evaluation of the effect of N fertilization on baking quality parameters remains difficult, since the S effect cannot be excluded from the evaluation.

## **4.5 Influence of sulfur fertilization on gluten composition and baking quality**

### *4.5.1 Assimilation and translocation of sulfur within the wheat plant*

Since S depositions from the atmosphere have decreased over the last few decades, the most important source of S for plants is the sulfate in soil, which is taken up by roots via sulfate transporters (Clarkson et al., 1993; Hawkesford et al., 1993; Buchner et al., 2004). Instead of assimilation in root cells, sulfate is, to a higher extent, transported to leaves for storage or direct assimilation (Larsson et al., 1991). Comparable to N assimilation, sulfate is stored in vacuoles as soon as sulfate uptake exceeds the rates of S assimilation. The first step of S assimilation is the activation of sulfate by ATP resulting in the formation of adenosine phosphosulfate. In the various subsequent steps, adenosine phosphosulfate is bound to thiol groups and is then reduced to sulfite ( $\text{SO}_3^{2-}$ ) and further to sulfide ( $\text{S}^{2-}$ ). All these assimilation steps are located in the chloroplasts of wheat leaf cells. In the next step,  $\text{H}_2\text{S}$  is incorporated into O-acetylserine to form the first S-containing amino acid, cysteine (Schiff and Saidha, 1987). Cysteine is further transported from chloroplasts to the cytosol to form methionine or is combined with glycine and glutamine to form the S-containing tripeptide glutathione (Ravanel et al., 1998; Hesse and Hoefgen, 2003). Glutathione fulfills several functions in plant

metabolism, such as the reduction of oxidative stress within the glutathione-ascorbate cycle (Noctor and Foyer, 1998) and the detoxification of xenobiotics (Schröder et al., 2007) and heavy metals (Cobbett and Goldsbrough, 2002). Additional functions concern S translocation in wheat plants. Glutathione is one component that is transported from vegetative tissues, predominantly the flag leaf, to the developing grain (Rennenberg et al., 1979). However, the component that is primarily transported to the grain is not yet completely known. The S-containing compounds sulfate, glutathione, and S-methyl-methionine have been found in phloem sap during grain development (Bonas et al., 1982; Larsson et al., 1991). However, in wheat, S-methyl-methionine is presumed to be the main transport form of S to developing grains (Bourgis et al., 1999). Grains seem to prefer S-containing metabolites for the formation of S-rich gluten proteins (Steinfurth et al., submitted). Another option involves the direct assimilation of sulfate that is taken up from the soil and then directly transported to the grain (Steinfurth et al., submitted). Nevertheless, grains are able to take up sulfate directly from the xylem stream which has been revealed by investigations on sulfate transporter expression in wheat plant tissues (Buchner et al., 2004). Recently, Buchner et al. (2010) have further elucidated the impact of vegetative plant tissues on the translocation of S within the plant during grain development. If the duration of grain development and storage protein synthesis is prolonged, an increasing number of leaves is responsible for maintaining S transport toward the grain. Young leaves and primarily the flag leaf are not able to meet the S demand of the grain, and therefore, older leaves have to mobilize S to supply the sink organ, viz., the grain.

Furthermore, the uptake and translocation of S is highly regulated. Endless absorption of S by the roots during vegetative growth is impossible, since sulfate transporters undergo a negative feedback mechanism (Saito, 2000). Nevertheless, the compound in plant metabolism that is responsible for the negative feedback on sulfate transporters is not yet known. Anderson and McMahon (2001) have assumed sulfate or glutathione to down-regulate the transporter expression. Additional investigations have revealed that glutathione alone is not able to repress the transporter expression. Thus, the ratio of sulfate to glutathione is suggested to act more probably as an internal signal (Rennenberg, 2001).

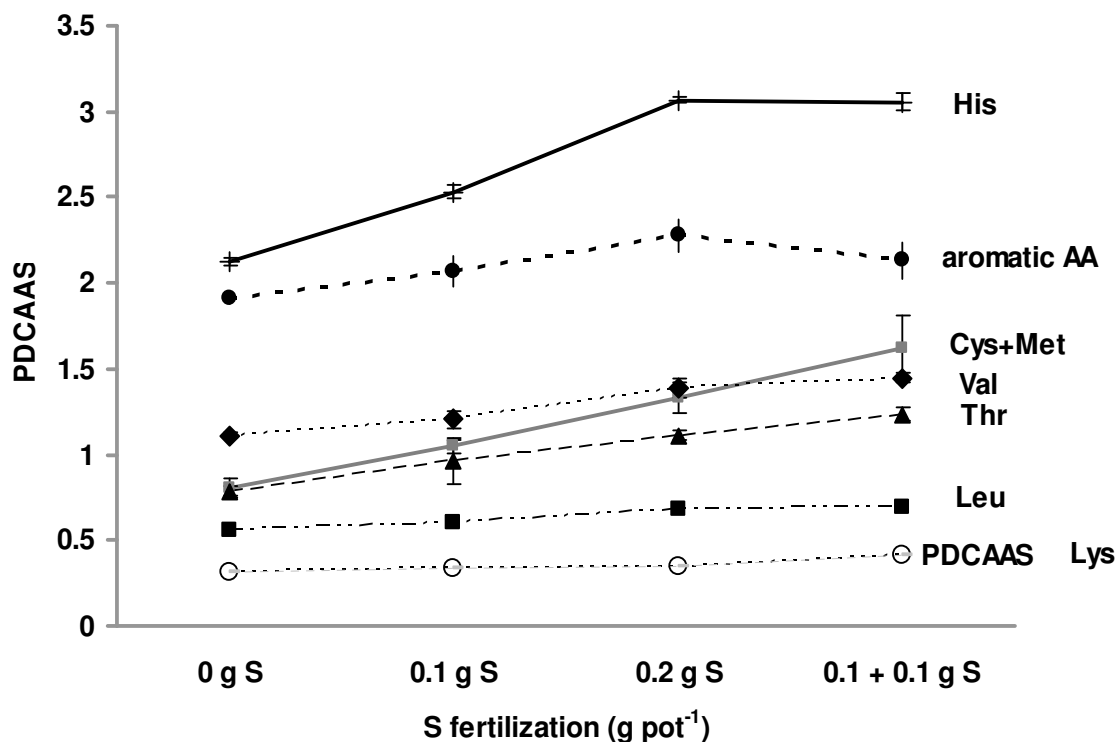
Grain usually only contains small amounts of sulfate (Steinfurth et al. submitted). Unlike vegetative tissues that contain about 50% of total S as sulfate, grains only contain about 1-5% of the total S as sulfate (Zhao et al., 1999a). Nevertheless, the way that wheat grain forms amino acids and polypeptides with a special function in bread making is of interest.

#### *4.5.2 Does sulfur fertilization increase gluten proteins as it has been reported for nitrogen fertilization?*

Increasing S fertilization is accompanied by a higher S concentration in grain (Zhao et al., 1999a; Zörb et al., 2009). High S amounts are incorporated into the S-containing amino acids cysteine and methionine, which accumulate in grain during development (Zhao et al., 1999a). Unlike replete S-supplied plants that predominantly store glutamate as a main amino acid in grain, S-starved plants accumulate the amino acid asparagine. This amino acid has devastating effects on the nutritional quality of bakery products. As the authors Granvogl et al. (2007) have shown acrylamide formation increases during the baking process since concentrations of free asparagine and sugars are enhanced because of S deficiency.

However, next to the negative effects of product processing, the protein quality of wheat is also highly important for human and animal nutrition since cereal products cover major amounts of daily protein intake (Max Rubner-Institute, 2008). To examine the biological value of each essential amino acid in wheat flour the protein digestibility corrected amino acid score (PDCAAS) was calculated (Schaafsma, 2000). For this purpose a pot experiment with increasing S fertilization (0 g S, 0.1 g S, and 0.2 g S at sowing) and, in particular, a late S fertilization (0.1 g S at sowing and additionally 0.1 g S at ear emergence) was conducted. This score has the advantage of evaluating the biological quality of protein by considering the characteristic digestibility and requirements for a 5-year-old child. The PDCAAS was not affected by S fertilization (Fig. 1). Unlike S fertilization, N fertilization decreases lysine concentrations (Shewry, 2007) because higher gluten concentrations are accompanied with a low lysine abundance (Kasarda et al., 1978). Nevertheless, the PDCAAS of cysteine and methionine increases with S fertilization (Fig. 1). Methionine is, in contrast to lysine,

not a limiting amino acid in wheat but is essential for human and animal nutrition. The facilitation of an adequately S-fertilized wheat might represent a chance to enhance the amount of methionine in the human diet and, hence, the protein quality consumed. Since humans and animals consume not only wheat-based products, high methionine levels in wheat can compensate for other low methionine sources.



**Fig. 1:** Impact of S fertilization on the biological value of wholemeal flour as calculated by protein digestibility corrected amino acid score (PDCAAS). Plant cultivation of cultivar Türkis was conducted according to Zörb et al. (2009). The cultivar indicated on the x-axis including a late S dressing (0.1 + 0.1 g S). PDCAAS was calculated according to Schaafsma (2000). His: histidine, arom. AA: aromatic amino acids Tyr+Phe, Cys+Met: cysteine+methionine, Val: valine, Thr: threonine, Leu: leucine, Lys: lysine.

Unfortunately, a further negative parameter concerns the correlation of the lysine concentration in wheat protein with baking quality. Nitrogen fertilization predominantly enhances the gluten protein concentration (Wieser and Seilmeier, 1998; Fuertes-

Mendizábal et al., 2010), but does not affect the albumin and globuline fractions. Since these fractions contain higher lysine amounts than gluten fractions (Shewry, 2009), increasing baking quality is negatively correlated with nutritional protein quality. However, the question arises as to whether S fertilization changes gluten composition, and whether these changes influence baking quality.

In freshly prepared flour, 95% of cysteine is present as inter- or intramolecular disulfides and only 5% as the thiol form (Shewry and Tatham, 1997; Grosch and Wieser, 1999). These disulfide bonds are predominantly formed between LMW and HMW glutenin subunits resulting in gluten-protein aggregates. Therefore, S fertilization changes the protein composition and baking quality mainly by increasing the cysteine concentration in gluten proteins. Unlike N fertilization that predominantly affects the quantity of protein in grain, S fertilization is known to “fine-tune” gluten proteins (Wieser et al., 2004; Zörb et al., 2009).

With respect to the Osborne fractions, the quantity of total gliadin and glutenin proteins is not affected by increasing S fertilization (Zörb et al., 2010). Unlike plants that receive no or little S, well-fertilized plants synthesize large amounts of S-rich  $\gamma$ -gliadin and LMW-glutenin proteins (Wieser et al., 2004; Zörb et al., 2009). Accordingly, S-poor gluten proteins such as  $\omega$ -gliadins and HMW-glutenins are reduced. However, how can the reduced synthesis of S-rich gluten proteins be explained by plant metabolism?

An adequate S supply during wheat plant development enhances the S pool in leaves and provides large amounts of S available for translocation to the grain. Furthermore, large amounts of glutathione are synthesized in the leaves (Steinfurth et al., submitted), and proteins might contain higher amounts of cysteine and methionine. Therefore, high concentrations of glutathione (Rennenberg, 1982) and S-methylmethionine (Bourgis et al., 1999) are translocated to the grain via the phloem. Exclusively under S deficiency, protein degradation in leaves is necessary to meet the high S sink capacity of grains (Fitzgerald et al., 1999). Because of high amounts of glutathione available in grain, S-rich proteins are primarily synthesized (Shewry et al., 2001). As long as large amounts of S are supplied to the grain, S-rich gluten proteins such as LMW glutenin subunits and  $\gamma$ -gliadins are predominantly synthesized (Wieser et al., 2004; Zörb et al., 2009). The

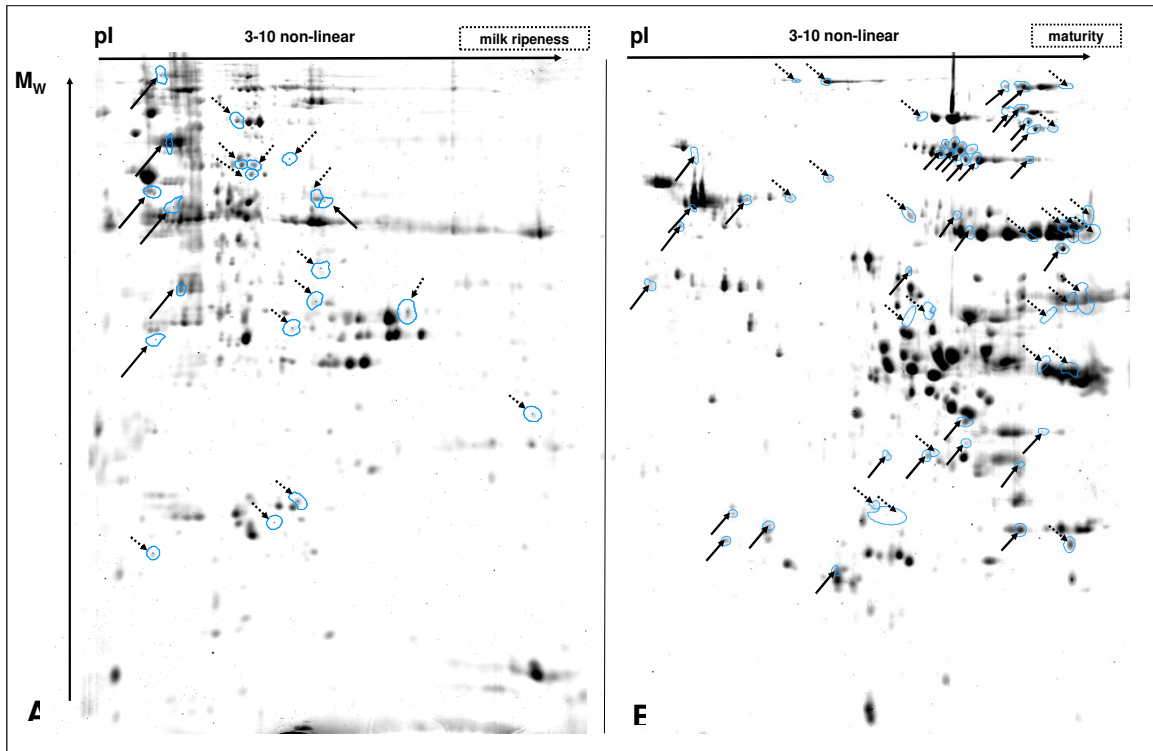
higher the S concentration of the grain, the higher the LMW glutenin concentration, and the lower the HMW glutenin concentration (Wieser et al., 2004). As a result, the HMW/LMW ratio increases. HMW glutenin subunits are thought to interact with other HMW or LMW subunits to form glutenin aggregates (Shewry et al., 1992). Several investigations concerning polymer formation have concluded that the associations between glutenin subunits are non-random. Glutenin subunits encoded by chromosome 1D are extensively involved in oligomer formation, and variants of genome 1B subunits differ in their ability to combine with 1A and 1D subunits (Lawrence and Payne, 1983). Furthermore, x-type subunits are able to form inter-chain bonds with two cysteine molecules, whereas y-type subunits use six thiol-groups for the formation of aggregates (Moonen et al., 1985). However, these aggregation models are still under discussion and need to be examined further in detail.

Higher S fertilization evidently leads to greater amounts of S being translocated to the grain, and therefore, a higher potential for aggregates in the dough. Without the aggregation of gluten proteins, wheat dough has no ability to gain volume during dough fermentation, since the matrix formation containing predominantly starch and gluten proteins is restricted (Gan et al., 1995). However, how does a late S fertilization or foliar application of S influence gluten composition? The impact of a late S fertilization on gluten composition has recently been investigated. The effects of late S fertilization on baking quality characteristics are highly controversial. Zörb et al. (2009) have observed no effects of late S fertilization on the protein and S concentration in wheat grain compared with grain obtained following fertilization with the same amount of S applied at sowing. Even more controversially, Luo et al. (2000) have concluded that a late S application is not necessary to optimize baking quality, but glutenin proteins respond to both late S and combined late N and S fertilization with quality reduction.

The experiment of Luo et al. (2000) has nevertheless a shortcoming concerning the N and S application time. Only the impact of N and S fertilization and its combination either at booting or at flowering has been examined. Hence, wheat plants might be grown on N- and S-deficient soil until booting and flowering, which has an important influence on plant metabolism in vegetative and generative tissues. To prevent quality losses, Haneklaus et al. (1995) concluded from S-starvation studies that S has to be supplied before S deficiency symptoms become apparent. Furthermore, Zörb et al.

(2009) have demonstrated that a late S fertilization at ear emergence prevents S deficiency attributable to low S application at sowing.

By comparing S fertilization at sowing with the split application of S, *viz.*, at sowing and at ear emergence, a late S application effectively results in an increased loaf volume (Selging et al., 2006; Zörb et al., 2009).



**Fig. 2:** Impact of S fertilization on protein synthesis in various developmental stages of wheat grain. Overlays of wheat flour proteins comparing high with late S fertilization. **A)** Overlay of images of two-dimensional (2D)-gel electrophoresis of immature milk ripe grain and **B)** overlay of flour of mature wheat grain. **Continuous** (rising) arrows illustrate the higher quantity of protein with fertilization levels after late S application, **dotted** (falling) arrows indicate higher protein quantity in plants with 0.2 g S at sowing. Wheat plants were grown under high (0.2 g S at sowing) and late S fertilization (0.1 g S at sowing + 0.1 g S at ear emergence). Kernels were harvested at milk ripeness and maturity. Pot experiment with cultivar Türkis and overlays of 2D gels comparing high S with late S fertilization were conducted according to Zörb et al. (2009).

Reversed phase-high performance liquid chromatography (RP-HPLC) and the 2D-gel electrophoresis of flour proteins might provide further insight into the alteration of gluten protein composition after late S fertilization. Zörb et al. (2009) have found



HMW glutenin subunits of type 1By9 and 1Bx7 to be enhanced after late S fertilization. However, Grove et al. (2009) have identified LMW glutenins and  $\gamma$ -gliadins as being enhanced after late S fertilization at early heading. Unfortunately, glutenins of high molecular weight were excluded from their analysis, because HMWs were negatively stained. In addition, late S fertilization changes the protein profile of wheat grain during whole grain development time. Figure 2 illustrates the changes in protein pattern of wheat grain with respect to milk ripe kernels (13 days post anthesis) and mature wheat grain. Grain material of milk ripe and mature kernels from a pot experiment with the winter wheat cultivar Türkis was harvested, and 2D-gel overlays of high S fertilization (0.2 g S at sowing) with late S fertilization (0.1 g S at sowing and 0.1 g S at ear emergence) were compared (Fig. 2). However, notably the protein profile predominantly changes in mature but not in milk ripe grain. In mature grain, more proteins are up-regulated in comparison with those in milk ripe grain. To conclude, S fertilization has an severe impact on the protein profile during the whole grain development and appears to change protein synthesis mainly during later stages of grain development. Shewry et al. (2009) have further illustrated protein synthesis during grain development by using 1D-gels and have demonstrated that LMW and HMW-GS are synthesized continuously until maturity, whereas other gluten proteins, such as  $\omega$ -gliadins do not eventually attain further concentration. Since protein synthesis occurs until grain maturity and continues during the dessication phase, changes in S fertilization appear to regulate gluten protein gene expression until maturity.

To date, further investigations, especially those under field conditions with diverse fertilization techniques involving soil or foliar application are necessary to elucidate completely the impact of a late S fertilization on gluten composition and baking quality characteristics. Foliar application of S, for example, is a strategy to prevent S deficiency in the late growth stages of wheat, since foliar applied S is still incorporated in grain gluten proteins (Tea et al., 2003). In addition to the impact of foliar S application on S translocation, Tea et al. (2005) have elucidated the impact of foliar S application on the thiol-disulfide status and polymeric protein formation in wheat grain. Glutathione is an interacting molecule in gluten formation during grain development (Huttner and Wieser, 2001; Tea et al., 2005). High levels of reduced glutathione (GSH) decrease the maximum resistance of dough and concomitantly increase extensibility (Kieffer et al.,

1990). Glutathione subsequently interacts with thiol-groups of gluten proteins and changes their properties. In particular, during the desiccation phase of grain development, the content of polymeric proteins increases. Furthermore, the impact of S fertilization on glutathione concentration and the polymerization of gluten proteins becomes noticeable during grain development (Tea et al., 2005). With low S fertilization, low polymeric protein-bound-glutathione is available and further low aggregated proteins emerge. Glutathione binds exclusively to cysteine residues that form inter-molecular disulfide bonds and are consequently responsible for the formation of LMW-GS aggregates. However, the effect of glutathione on gluten composition and its results in baking quality are not yet completely understood. Further investigations are needed. Reduced glutathione is known to affect the rheological properties of dough by the depolymerization of glutenin polymers via the specific cleavage of inter-molecular disulfide bonds. Reinbold et al. (2008) have elucidated the effect of increasing S fertilization on the thiol and glutathione status of S-deficient wheat. Since the dough made from S-deficient flour is highly resistant to expansion, it is of interest to determine the way that S fertilization changes SH-groups in wheat flour. With increasing S fertilization, cysteine and glutathione levels are enhanced. These authors have revealed that, in S-deficient wheat cysteine and glutathione correlated with bread volume in the same extent as the protein concentration. So far, S fertilization is known not only to enhance glutathione concentration in wheat flour, but concomitantly also the amount of S-containing gluten proteins. There is a need to elucidate further whether the impact of glutathione on gluten proteins is different between low S plants and high S plants.

#### *4.5.3 Is there a potential to increase baking quality further by adequate sulfur fertilization?*

Whereas an examination of the individual impact of N fertilization on baking quality parameters is difficult, the impact of S fertilization on baking quality has been demonstrated in several investigations. So far, S concentration in grain alone is not able to explain all the impact on loaf volume. An increase of the S concentration in grains, accompanied by adequate N fertilization, increases the extensibility of dough while doughs become less short and resistance decreases (Kettlewell et al., 1998; Zhao et al., 1999b; Flæte et al., 2005). Accordingly, S deficiency leads to high resistant doughs with

low extensibility (Wrigley et al., 1984). These quality characteristics account for the higher potential of doughs to prove during dough fermentation because of the disulfide bonds in the gluten structure (Shewry et al., 1992), resulting in higher loaf volume (Moss et al., 1981; Flæte et al., 2005) among adequate S fertilization. Zhao et al. (1999b) have shown a positive but low correlation of S concentration in grain, with loaf volume. Therefore, grain S concentration more clearly explains an increasing dough extensibility. Furthermore, the gel-protein also called glutenin macro polymer, which is difficult to extract from wheat flour because of its high aggregation behavior, has a higher correlation with dough extensibility than the S concentration by itself. Haneklaus et al. (1992) have also been able to explain 40% of the variability in loaf volume based on grain S concentration. Reinbold et al. (2008) have further found cysteine and glutathione concentrations in S-deficient wheat to be positively correlated with  $R_{max}$ , extensibility, and extension area. Thus, these data confirm the way in which thiol groups and glutathione interact with S-rich gluten proteins and explain the changes in baking properties. As another important aspect, late S fertilization is considered to affect baking quality positively. Seling et al. (2006) have enhanced RMT loaf volume of winter wheat with a late S fertilization by 117 mL 100 g<sup>-1</sup> flour. The enhanced baking quality may be referred to an increase of HMW subunits 1Bx7 and 1By9 glutenins found to be increased after late S fertilization (Zörb et al., 2009). This combination of proteins was formerly demonstrated to have a strong positive relationship with the Zeleny sedimentation value and dough resistance and therefore with high baking quality (Branlard and Dardevet, 1985; Seilmeier et al., 1991).

Tea et al. (2007) have examined the effect of foliar N and S application as a late application at anthesis after soil N treatment with ammonium nitrate during vegetative growth. Flour protein content was increased each with N and S treatments. However, combined N and S foliar applications resulted in the highest protein concentration. Alveograph measurements confirmed previous studies with soil-applied fertilizers. Nitrogen or combined N and S treatments increased dough strength, whereas S and combined N and S treatments increased dough swelling and extensibility. Hence, foliar N and S application at anthesis is an alternative to soil application during late wheat development, with applied nutrients still being assimilated and incorporated into gluten proteins.

An increase in the quantity of gluten proteins leads to a higher potential of dough to expand during dough fermentation, since more gas cells develop during mixing surrounded by the characteristic starch-protein-matrix (Gan et al., 1995). Furthermore, cysteine residues within this protein-matrix are able to assure extensibility and lower resistance. A balance between extensibility and resistance is of particular importance, since excessive resistance of dough limits expansion, and excessive elasticity leads to a failure of the dough to retain CO<sub>2</sub> during fermentation (Zhao et al., 1997). An adequate relationship of N and S has therefore to be maintained for adequate baking results, and further increases in baking quality are possible by the addition of adequate S fertilization concomitantly with adequate N fertilization.

#### **4.6 The way that nitrogen and sulfur interact in gluten composition**

A range of investigations highlights the importance of the adequate management of concomitant N and S fertilization. For example, Zörb et al. (2010) have found total protein and as well gliadin and glutenin concentrations to increase in flour following the N fertilization of wheat. In addition, a concomitant increase in S fertilization has no effect on total protein concentrations. However, increasing N fertilization without adequate S fertilization can provoke S-deficiency symptoms, despite an increase in yield and protein concentration. Moreover, Eriksen and Mortensen (2002) have examined the effect of S fertilization on barley; S-containing amino acids cysteine and methionine increase following S fertilization, but N concentration decreases because of a dilution effect caused by increased yield. Hence, baking quality can only be enhanced by increasing N fertilization levels providing that the S abundance in proteins is adequate. The question is: what exactly is meant by "adequate"? Randall et al. (1981) and Zhao et al. (1999a) have stated that N/S ratios of 17:1 and higher in wheat grain indicate S deficiency. Loaf volume can be enhanced by N fertilization at first, but as soon as the N/S ratio increases to inadequate levels, S fertilization needs to be adjusted. On the contrary, the S concentration in grain depends on the protein concentration, since the abundance of N and S in gluten protein subunits is genetically manifested. A high S concentration in grain consequently occurs when both high N and high S supply is applied (Moss et al., 1981). Throughout plant development the abundance of metabolites following N and S assimilation determines the expression of individual

gluten protein genes (Tabe et al., 2002). Therefore, splitting the rates of N and S or of different fertilizer forms, such as soil or foliar applications, are alternatives to guarantee nutrient availability throughout plant and grain development. Fast mobilization and retranslocation rates within the plant enable the reliable assembly of late-applied N and S into storage proteins and lead to high quality gluten protein properties during breadmaking.

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## **Chapter 5**

### **Comparison of Baking Tests Using Wholemeal and White Wheat Flour**

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*submitted*

## **5. Comparison of baking tests using wholemeal and white wheat flour**

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## 5.1 Abstract

Production of whole-grain products can provide more variety in bakery products and might impact positive health effects due to fermentable soluble fibers. To assess technological quality micro-scale methods using 10 g of white flour are usually carried out, in particular if amount of material or additives is limited. Using wholemeal flour for micro-scale testing might enhance use efficiency of raw material. Up to date, there is no information, whether micro-scale testing of wholemeal flour provides the same information on techno-functionality as classical methods. Two wheat varieties with the same protein concentration but different bread making performance were used to analyze this aspect. Micro-scale baking test was optimized for wholemeal flour concerning dough consistency and fermentation time. Wholemeal flour reduced loaf volume but optimization was successful. Although all processing steps of wholemeal baking test were adapted, differences in loaf volume between the two varieties were only present with white flour. Coarse particles in wholemeal flour may be responsible for reduced gas retention. Decreasing particle size of bran material might reduce adverse effects on loaf volume. A micro-scale baking test using wholemeal flour would have several advantages after correct implementation: lower price, less time and material required, direct measurement of quality aspects.

**Key words:** wholemeal flour, micro-scale baking test, white flour, rapid-mix-test

## 5.2 Introduction

Bread and bakery products are the basis of human nutrition worldwide. Beside its main function as starch and energy supplier, bread and bakery products offer high amounts of vitamins, minerals, trace elements and dietary fiber. As nutritionally beneficial constituents are primarily located in the germ and bran (AACC, 1999), wholegrain cereal products are enriched in these compounds. Additionally, a low glycaemic index of whole grain can lower the risk for chronic diseases. Starch in breads baked with fine flour evokes a rapid increase of plasma glucose, while after consumption of wholemeal products the increase of the concentration of postprandial glucose is lowered (Jenkins et al., 2002). This can lead to a decreased risk for diabetes type 2. However, in many western countries the consumption of whole grain foods is estimated to be too low (Cleveland et al., 2000; Thane et al., 2005). Tremendous efforts are made to enhance the consumption of whole grain food. One example is the use of health claims on functional food or research on consumer behavior concerning whole grain products (Arvola et al., 2007; Dean et al., 2007; Vassallo et al., 2009). Wholemeal bread is highly considered as a functional food carrier, however, there is still a limited selection of products that meet consumers' expectations (Adams and Engstrom, 2000) and adequate food process strategies (Camire, 2004). To overcome this situation, the development of suitable methods to measure the functionality of wholemeal flours is required. So far, no baking tests with wholemeal flour are used in research and industry as routine methods. Therefore, efforts were made to study the suitability of existing standard baking tests for white flour also for wholemeal flour. The rapid-mix-test (RMT), a standard test using 1000 g of white flour, was suggested to be also suitable for wholemeal flour (Jahn-Deesbach et al., 1989). However, no studies on adapting the RMT to wholemeal flour are available up to now.

For research studies with a limited amount of material (flour, fractions or additives) micro-scale baking tests on the basis of 10 g of white flour have been developed (Kieffer et al., 1993), which have been termed micro-scale baking test (MBT; mixing in the micro-farinograph) and micro-rapid-mix-test (MRMT; high-speed mixing). However, wholemeal flour has not been used with these methods up to now. Therefore, the aim of this study was to develop a micro-scale baking test (MBT) on the basis of 10 g wholemeal flour as a tool to determine the baking performance of wheat without

the need to prepare white flour. The suitability of the new MBT should then be compared with the performance of the RMT and the MBT carried out with white flour.

### **5.3 Material and Methods**

#### *Wheat grain and wheat flour*

Wheat grain of the cultivars (cv.) Batis and Türkis was obtained from the German Federal Office of Plant Varieties out of five different field trials in Germany. These varieties were chosen due to their quality aspects. The varieties are classified in the same baking quality class and differ with respect to quality characteristics only in terms of loaf volume with cv. Türkis accounting for a higher loaf volume as compared to cv. Batis. In terms of crude protein concentration, sedimentation value and dough elasticity both cultivars exhibit comparable quality characteristics. According to these parameters the suitability of different baking tests for quality assessment could be studied. Prior to milling, grains were adjusted to 14 % moisture content. To obtain white flour, grains were processed according to a German standard milling procedure (Association of Cereal Research, (1994). Processing resulted in a flour ash concentration of 0.6 % in dry mass as determined by ICC method No. 104/1 (International Society for Cereal Science and Technology ICC (1994)) and sifted through a 200 µm sieve. To produce wholemeal flour the grains were milled on a Titan laminated mill using a 500 µm sieve (Retsch, Haan, Germany). The flour was allowed to rest for up to two weeks prior use. Nitrogen content was determined using the method of Dumas (International Society for Cereal Science and Technology (ICC), 1994); method No. 167) on a Leco FP 2000 (Leco, Mönchengladbach, Germany).

#### *Water absorption of flour*

Water absorption of flour was determined in a micro-farinograph (Brabender, Duisburg, Germany). 10 g of flour, 0.2 g NaCl, 0.2 g sucrose, 0.1 g coconut fat, and water were mixed at 22 °C for 20 min. The amount of water was adjusted to give maximum dough consistencies of 450, 500, and 550 Brabender Units (BU), respectively.

### *Micro-scale baking test*

The MBT with white flour was performed according to Kieffer et al. (1998). Fermentation time of cv. Türkis was adjusted to 35 min because of reduced loaf volume at 40 min of standard fermentation time. To optimize dough texture the wholemeal rolls were baked with a dough consistency of 450, 500 and 550 BU. The wholemeal dough was mixed in a 50 g farinograph mixing bowl (Brabender, Duisburg, Germany) at 60 rpm and divided into five equal parts before the first proofing. For optimization of fermentation time the rolls were fermented for 10-30 min at 30 °C in a water-saturated atmosphere. Finally, for better comparison of RMT and MBT (white and wholemeal flour) malt flour was used to adjust falling number to 250 s.

### *Rapid-mix-test*

The RMT was performed according to Association of Cereal Research (1994)

### *Data, replication, statistics*

All statistical analysis was carried out using SAS (SAS Institute Inc., Cary, USA, Release 9, 2001). Comparisons of means with respect to the influence of the cultivar (cv. Batis and cv. Türkis) were carried out using the t-test. Statistical significance ( $p < 0.05$ ) is indicated by capitals linked to individual data points. All determinations were carried out in duplicates.

## **5.4 Results**

To compare the MBT with the standard-scale baking test (RMT), white flours from five different field trials in Germany were processed under standard conditions. Due to high standard errors no statistically significant difference in loaf volume between cv. Batis and Türkis was found with the RMT (Fig. 1A). Because of highly variable environmental conditions on the fields, the protein concentration of the varieties was different. To compensate for the high influence of the protein concentration on the loaf volume, the specific loaf volume (loaf volume per % protein concentration) was calculated (Fig. 1B). This eliminated the variations and provided statistically significant differences between cv. Batis and Türkis in the RMT (Fig. 1B). The difference in

specific loaf volume determined by the RMT ranged between 53.6 mL per 100 g flour and 58.0 mL per 100 g flour for cv. Batis and Türkis, respectively. Differences in loaf volume determined by the MBT with white flours ranged from 45.1 mL per 10 g flour to 51.9 mL per 10 g flour for cv. Batis and Türkis, respectively (Fig. 2A).

To use wholemeal flour in a micro-scale baking test several parameters had to be adjusted during the process. First, the impact of dough consistency on the loaf volume was tested by preparing doughs with three different consistencies (Fig. 3). For white flour optimum bread making performance was found with a maximum consistency of 550 BU. When wholemeal flour was used, no impact of dough consistency on loaf volume was found (Fig. 3). Second, the impact of fermentation time after rounding on loaf volume had to be adjusted. In the MBT with white flours of cv. Batis and Türkis fermentation times of 40 and 35 min gave the best results, respectively. With wholemeal flours the fermentation time strongly influenced the loaf volume (Fig. 4). Differences in loaf volume after variation of fermentation time ranged between 31.0 ml per 10 g flour and 34.0 ml per 10 g flour and between 28.0 ml per 10 g flour and 32.0 ml per 10 g flour over a time period of 30 min for cv. Batis and Türkis, respectively (Fig. 4). Unexpectedly, cv. Türkis had lower loaf volumes than cv. Batis. Due to this fact malt meal was added in all further experiments to adjust falling number to 250 s. A fermentation time of 20 min was found to give the highest loaf volumes, whereas longer fermentation times led to a decrease of the loaf volumes. Wholemeal flours absorbed more water during the kneading process and had higher protein concentration compared to the white flour (Tab. 2). Finally, a process for bread making with wholemeal flour was conducted according to Tab. 1. A dough consistency of 500 BU was used because it provided the best processability of the dough. The MBT with white flour gave higher loaf volumes than the MBT with wholemeal flour. Loaf volumes were increased by 28 % and 37 % for cv. Batis and Türkis, respectively, when white flours instead of wholemeal flours were used for baking (Fig. 2A and B). In contrast to the MBT with white flour the MBT with wholemeal flour provided no significant differences in loaf volume between the two cultivars (Fig. 2B; 32.3 mL per 10 g flour and 32.9 mL per 10 g flour for cv. Batis and Türkis, respectively). The same as in the MBT with white flour the specific loaf volume was calculated for the wholemeal baking test to eliminate the impact of the protein concentration on the loaf volume. However, there was no

significant difference between the specific loaf volumes of cv. Batis and Türkis (Fig. 2C).

## 5.5 Discussion

Two different wheat cultivars were assembled to obtain data on baking quality using standard-scale and micro-scale baking tests with white and wholemeal flour. The cultivars Batis and Türkis were selected because they had different bread making performance in spite of similar protein concentration, sedimentation value and dough elasticity. Cv. Türkis has been described to form a higher loaf volume than cv. Batis on the basis of the same protein concentration (2005). Since all quality parameters of the two cultivars except the loaf volume were comparable they were selected to study the influence of the type of flour (white or wholemeal) on the loaf volume.

### 5.5.1 Comparison of standard-scale and micro-scale baking tests

The RMT is a standard-scale baking test using 1000 g of white flour to obtain direct results on the baking quality of wheat. For research purposes the high amount of flour needed for the RMT is a problem due to often limited access to sample material. To proof direct impacts of plant nutrients on wheat quality, pot experiments are often used but they provide insufficient material for the RMT (Zorb et al., 2009). Furthermore, to examine the influence of flour additives and extracted protein fractions on baking quality it is more efficient and cheaper to use small-scale tests (Wieser and Kieffer, 2001). Therefore, Kieffer et al. (1993) developed a micro-scale baking test with only 10 g flour. This test was carried out in two versions, the micro-scale baking test (MBT), in which the dough was mixed in a farinograph and the micro-rapid-mix-test (MRMT) with high-speed mixing to mimic the RMT. In this study the MBT was used for white flour as well as for wholemeal flour.

The superior bread making performance of cv. Türkis was confirmed by both the RMT (Fig. 1) and the MBT with white flour (Fig. 2A). However, the difference in specific loaf volume was higher in the MBT than in the RMT (12 % vs. 8%; data not shown). Thus, the MBT had a higher potential to determine differences between wheat cultivars.

5.5.2 *Bread making performance of white and wholemeal flour*

The different bread making performances of cv. Batis and Türkis found with the RMT and MBT with white flour were no longer present in the MBT with wholemeal flour (Fig. 2). Additionally, the loaf volumes were smaller for wholemeal flour than for white flour although the former had higher protein concentration than the latter (Tab. 2). Reasons to explain problems in breadmaking with wholemeal flour are multi-causal. At first, this aspect can be explained by different protein compositions of white and wholemeal flours. Gliadins and glutenins are storage proteins that are only present in the endosperm. They have a major impact on the bread making performance and are enriched in white flour as compared to wholemeal flour. Instead of gliadins and glutenins the bran and aleurone layers contain albumin- and globulin-like proteins, which are regarded as remnants of the cytoplasm and other subcellular fractions. The effect of albumins and globulins on baking quality is not yet clearly examined (Shewry, 1996).

A second reason for the poorer bread making performance of wholemeal flour are bran particles present in the flour. To understand the detrimental effect of bran on loaf volume gas cell processes during mixing, proofing and baking have to be considered. Gas cell nuclei are incorporated into the dough by occlusion of air during mixing (Baker and Mize, 1946) while the yeast is incapable of forming new gas cells. This means that mixing is one critical point and should generate as much gas cell nuclei as possible. This primary number of gas cells is surrounded by a starch-protein-matrix at advanced stages of proofing. At this stage the homogeneity of protein strands and protein films play a crucial role in gas holding capacity. Furthermore, discontinuities in the matrix form inter-connections between adjacent cells (Gan et al., 1990). But these gas cells remain separated by a lamellar liquid film. Both, a stable starch-protein-matrix and a stable lamellar liquid film are essential for gas retention (Gan et al., 1995). During processing of wholemeal products the fiber and bran particles in the flour cause discontinuities and in homogeneities of strands, films and membranes and decrease gluten strength and gas holding capacity leading to a lower loaf volume. The reduction of loaf volume by bran or fiber is also described in several publications (Gan et al., 1992b; Jahn-Deesbach et al., 1989; Rogers and Hosney, 1982; Wang et al., 2002).

In the 1980s several investigators examined whether it is possible to use wholemeal flour in a RMT (Jahn-Deesbach et al., 1989; Zwingelberg et al., 1985). The particle size of flour had a high impact on loaf volume. While wholemeal flour with a particle size of < 180  $\mu\text{m}$  produced the highest loaf volume, coarser flour (particle size 3000  $\mu\text{m}$  and 1500  $\mu\text{m}$ ) led to lower loaf volumes. The method was capable of determining the bread making performance of several wheat varieties but it has not been used in the future.

Another aspect explaining the poorer breadmaking performance of wholemeal flour is the higher water absorption of wholemeal flour (Tab. 2). Dietary fiber increases the water absorption because the high number of hydroxyl groups existing in fiber allows more water interactions due to hydrogen bonding (Rosell et al., 2001) as compared to white flour. Higher fiber content with high water absorption capacity leads to a dilution of technologically active proteins within the dough (Van Hung et al., 2007; Wang et al., 2002). Lower protein concentration decreases the loaf volume and, as a second aspect, negatively affects the formation of a viscoelastic network of cross-linked gluten protein components (Van Hung et al., 2007). Easy disruption of the gluten network is a consequence resulting in low gas holding capacity and low loaf volume. Gan et al. (1992a) examined, which parts of the whole grain affect the baking performance and studied the influence of outer layers from different wheat grists on loaf volume. One fraction with epicarp hairs was found to be the main factor reducing the loaf volume more than outer bran fractions. Epicarp hairs disrupt the starch-protein-matrix and restrict gas cells to expand. Enzymes of outer bran fractions were detected to have no adverse effects on loaf volume. A last aspect of decreased loaf volume by wholemeal fractions in the dough is only a slight oven-spring caused by early fixing of the structure and a high water content of the dough during baking (Rogers and Hosenev, 1982). All these aspects on dough formation and gas cell expansion caused a decreased loaf volume with wholemeal flour as shown in Fig. 3A and B).

The adaptation of the MBT to the use of wholemeal flour was successful. The bran particles had no adverse effect on mixing in the micro-farinograph, wholemeal doughs were easy to handle during rounding and proofing and developed a continuous crumb structure during baking. However, there is still the question why it was not possible with wholemeal flour to distinguish between the bread making performance of the two wheat cultivars as this was possible with white flour. An explanation for this could be



thousand kernel weight, which differs between cv. Batis and Türkis. According to the German Federal Office of Plant Varieties (2005) cv. Türkis has a higher thousand kernel weight compared to cv. Batis. Therefore, a fixed amount of wholemeal flour from cv. Türkis contains more bran particles than from cv. Batis. Thus, more detrimental effects of bran particles during bread making can be expected for cv. Türkis as compared to cv. Batis.

Flour particle size might also be the reason for the lower loaf volumes observed, affecting the MBT with wholemeal flour. Dreyer et al. (1985) used wholemeal flour with finer particle size of  $< 180 \mu\text{m}$  instead of  $< 500 \mu\text{m}$  in their study. Based on these results it can be assumed that the differences between the baking tests with white and wholemeal flour might disappear, if a smaller particle size of wholemeal flour (e.g.  $< 180 \mu\text{m}$ ) would be used.

### 5.5.3 *Future perspectives*

Breads and wheat products containing whole grain, multi-grain or other functional ingredients are becoming more important in the bakery industry due to a higher awareness of a healthy lifestyle (Martin, 2004). The production of multi-grain products can provide more variety in breads and wheat products and increases the diversity in fermentable soluble fibers (Lopez et al., 2001), which have positive effects on people's health. However, studies for an increase of the nutritional value of bread and wheat products rise technological issues because healthy and fiber-rich bakery products will only have a chance in the marketplace, if they have good texture and good taste at the same time (Dewettinck et al., 2008). Milling and processing technology of cereals can decrease or increase levels of beneficial bioactive compounds (Slavin et al., 2001). In the same way, technology might also affect (techno-) functional properties to produce wholemeal flours, which could be studied for bread making performance using standardized baking tests that yield the same results as baking tests with white flour. E.g. the use of different milling fractions may be helpful to decrease adverse effects of coarse bran particles in wholemeal flour. Dreyer et al. (1985) used different milling fractions by using several wholemeal flour types in the RMT. Bran has impact on loaf

volume, but source of bran and particle size of bran is essential. Lai et al. (1989) and Zwingelberg et al. (1985) determined the source of bran and the particle size of bran to be key factors that influence loaf volume. Consequently, fine grinding of bran resulted in higher loaf volumes.

## **5.6 Conclusions**

Micro-scale test methods using wholemeal flour can contribute to reliable test methods for baking quality that are low-priced compared with the RMT, less time and material consuming, and allow the direct use of wholemeal flour for quality testing. Conventional micro-scale baking tests can be optimized for using wholemeal flour concerning dough texture and fermentation time. Particle size of the flour is a key parameter for the (techno-) functionality of wholemeal flour. Optimization of flour milling to yield small bran particles may reduce adverse effects of fiber and bran on loaf volume.

## **Acknowledgements**

We appreciate the excellent technical assistance of Mrs. Katharina Schiesser.

Figures

Fig 1

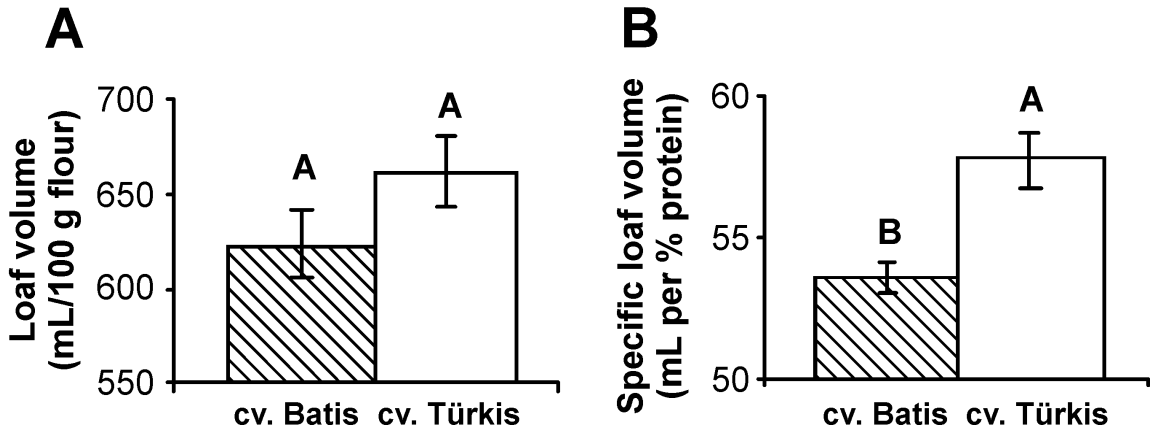


Fig 2

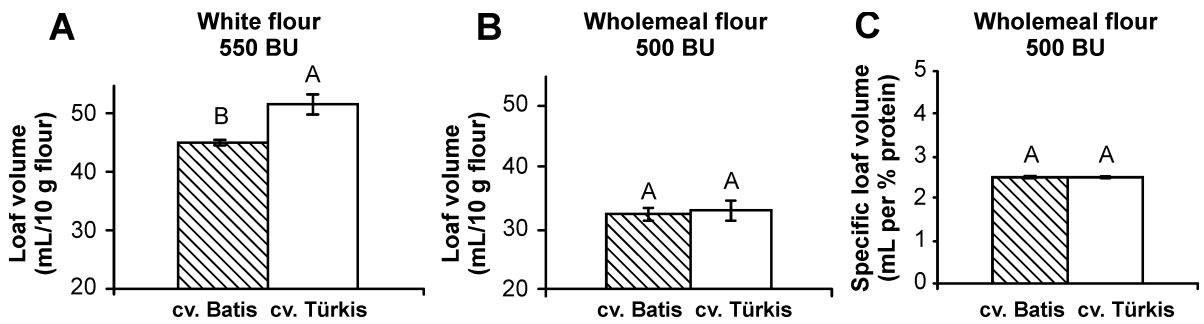


Fig 3

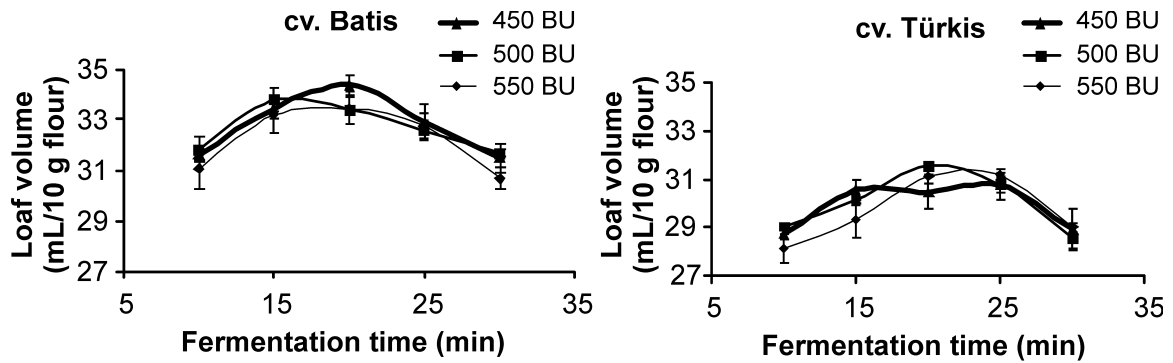
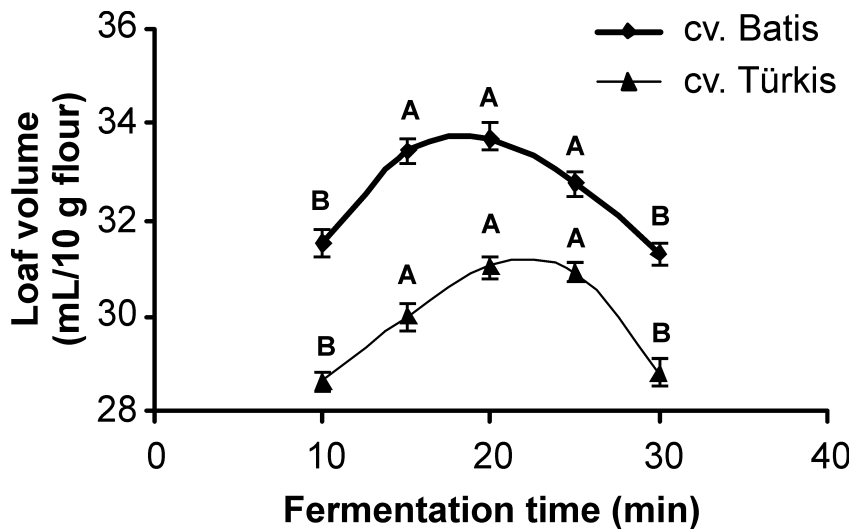


Fig 4



## Figure captions

Fig. 1: **A** Loaf volumes of white flours of cv. Batis and Türkis in the standard-scale baking test (RMT) with 1000 g of flour and **B** corresponding specific loaf volumes (loaf volume per % protein). Doughs had a consistency of 550 BU. Error bars represent  $\pm$  standard errors of three independent field replications. Different capital letters given with the data represent statistically significant ( $p \leq 0.05$ ) differences.

Fig. 2: Effect of flour type on loaf volume in micro-scale baking tests using white and wholemeal flours of cv. Batis and Türkis. Falling number was adjusted to 250 s prior to baking **A** Loaf volumes in the MBT according to Kieffer et al. (1993; 1998) with white flour.. **B** Loaf volumes in the MBT with wholemeal flour. **C** Specific loaf volumes (loaf volume per % protein). Error bars represent  $\pm$  standard errors of three independent field replications. Different capital letters given with the data represent statistically significant ( $p \leq 0.05$ ) differences.

Fig. 3: Influence of the fermentation time and dough consistency on the loaf volume of cv. Batis and Türkis in the MBT with wholemeal flour. Error bars represent  $\pm$  standard errors of three independent field replications.

Fig. 4: Effect of fermentation time on loaf volume of cv. Batis and Türkis as determined by the MBT with wholemeal flour. Error bars represent  $\pm$  standard errors of three independent field replications. Statistical significance ( $p \leq 0.05$ ) is indicated by capital letters for the fermentation time.

## Tables

Tab 1

	RMT	MBT (white flour)	MBT (wholemeal flour)
<b>Flour</b>	1000 g	10 g	10 g
<b>Mixing and dough texture</b>	Stephan kneader 20 + 40 sec (500 BU)	Farinograph Until dough optimum (550 BU)	Farinograph Until dough optimum (500 BU)
<b>Dough temperature</b>	26-27 °C	22 °C	22 °C
<b>1<sup>st</sup> Dough maturing</b>	Proofing cabinet 20 min	Proofing cabinet 10 min	Proofing cabinet 10 min
<b>1<sup>st</sup> Rounding</b>	Manual	Homogenisation Machine-made	Homogenisation Machine-made
<b>Proofing of bales</b>	In the room 10 min	None	None
<b>Separation</b>	Machine-made Differential pressure 5,5-6,5	None	None
<b>2<sup>nd</sup> Rounding</b>	12 U	None	None
<b>Stretching</b>	Machine-made	Machine-made	Machine-made
<b>Wrapping</b>	Machine-made	Manual	Manual
<b>Fermentation</b>	Proofing cabinet 25 min	Proofing cabinet 40 min	Proofing cabinet 20 min
<b>Drying and Conversion</b>	2 min.	None	None
<b>Baking</b>	18 min, 230 °C	10 min, 230 °C	10 min, 230 °C

Tab. 2

Harvesting year	Site	Variety	Protein content [%DM] (refined flour)	Protein content [%DM] (wholemeal flour)	Loaf volume RMT [mL/100g] (refined flour)	Flour water absorption [%] RMT	Flour water absorption [%] (refined flour)	Flour water absorption [%] (wholemeal flour)
2009	7901	Batis	10.5	11.4	589	57.9	58.0	62.0
2009	7902	Batis	12.2	13.3	660	58.3	57.5	67.0
2009	7903	Batis	12.7	13.5	653	57.0	56.0	68.2
2009	7904	Batis	12.7	13.6	676	58.7	59.5	69.2
2009	7905	Batis	10.9	11.6	584	63.6	61.0	66.0
2009	7901	Türkis	10.8	11.9	632	57.2	61.7	61.7
2009	7902	Türkis	10.8	11.9	601	58.4	65.0	65.0
2009	7903	Türkis	12.6	13.6	722	58.0	65.5	68.7
2009	7904	Türkis	13.1	14.2	717	58.7	66.0	69.0
2009	7905	Türkis	10.5	11.7	642	60.4	62.5	65.2

### **Table captions**

Tab. 1: Procedures of the RMT and the MBT with white flour according to Kieffer et al. (1993) and the MBT adapted to wholemeal flour.

Tab. 2: Protein concentration (%) in dry matter, loaf volume determined by RMT and water absorption (%) of white and wholemeal flour of cv. Batis and Türkis. Flour water absorption was in two separate determinations for the RMT (Association of Cereal Research, 1994) and for the MBT (International Society for Cereal Science and Technology (ICC), 1994) (micro-farinograph) (Kieffer et al., 1998).

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## **Chapter 6**

### **General Discussion**

## **6. General Discussion**

There are a few studies that have examined the impact of N and S fertilization on both, storage protein composition and baking properties concomitantly. Furthermore, there are investigations that demonstrate an improved baking quality after a late S fertilization, but no information about storage protein changes are available (Seling et al., 2006). So far, there is still a lack of knowledge concerning the above mentioned aspect. This thesis aimed to enlighten the cascade of plant metabolism influenced by S fertilization and in particular a late S fertilization, storage protein composition and baking quality.

### **6.1. Influence of sulfur fertilization on storage protein composition**

Late S fertilization (0.1+0.1 g S) was shown to change protein composition in mature wheat grain (Zörb et al., 2009) (chapter 3, fig. 5 and 6). However, it is interesting to know, how plant metabolism is affected by late S fertilization, as well.

There are two potentialities to take up S into developing wheat grain: First, direct uptake from soil via the xylem (Buchner et al., 2010), and second, remobilization of S-containing compound from the flag leaf and translocation via the phloem (Rennenberg et al., 1979; Bourgis et al., 1999). Therefore, the distribution of S-containing compounds such as proteins, sulfate, and glutathione in several wheat plant tissues were analyzed to show how S is distributed during grain development (chapter 2, fig. 1, 3 and 4). Late applied S is rather transported to the flag leaf in the form of sulfate than directly transported to developing ears. In the flag leaf sulfate is assimilated and later transported as glutathione to the developing grain. However, during milk ripeness amounts of glutathione in the flag leaf, ears and milk ripe grain are equal comparing high with late S fertilization. So far, plant tissues convert late applied S at ear emergence into glutathione within a few days to supply the developing grain. Therefore, it is possible to apply late S at ear emergence, since plant tissues compensate for low amounts of S in former plant developmental stages (chapter 2).

Although late S fertilized grain contained equal protein and S concentrations compared to plants that obtained the same amount of S (0.2 g S) only in one dressing at sowing,

loaf volume was enhanced (Zörb et al., 2009) (chapter 3, fig. 2, 4 and 7). Since the quantity of protein is obviously not crucial to explain differences in baking quality, there might be a change in individual proteins. It was possible to illustrate these changes in protein composition by 2D-gel electrophoresis (Zörb et al., 2009) (chapter 3, fig. 6 and chapter 4, fig. 2).

To further obtain more detailed information about the different S-poor and S-rich gliadin and glutenin subunits changed after late S fertilization, analysis of fractionated protein subunits according to a method provided by Wieser et al. (1998) on RP-HPLC were conducted. As Wieser et al. (2004) reported, S-rich protein subunits like LMW-GS and  $\gamma$ -gliadins were synthesized at higher amounts than the S-poor protein subunits HMW-GS and  $\omega$ -gliadins. In accordance to total protein concentration, total gliadin and glutenin fraction, except the non S treatment, were not affected by S fertilization resulting in equal concentrations (Zörb et al., 2009). The wheat grain therefore compensates a lower S supply during grain development. This compensation results from a synthesis of S-poor gliadin and glutenin subunits (Wieser et al., 2004). However, with respect to late S fertilization the cultivars, Batis and Türkis, showed heterogeneous changes in S-rich and S-poor gliadin and glutenin subunits (chapter 3, fig. 5). Both cultivars synthesized higher amounts of  $\gamma$ -gliadins after late S fertilization, but were different in the synthesis of other gliadin subunits. With respect to glutenin subunits Batis synthesized more LMW-GS, but evinced no changes in HMW-GS. However, Türkis had the same amounts of LMW-GS comparing high with late S fertilization, and HMW-GS decreased under late S fertilization (Zörb et al., 2009) (chapter 3, fig. 5). To conclude, by RP-HPLC it was not possible to find the same changed proteins in both cultivars after late S fertilization explaining the increased loaf volume. Therefore, identification of changed protein spots per MALDI-TOF was used to obtain information, first, about changes of protein composition in both cultivars, and second, about a possible function in wheat metabolism. Four protein spots matched with hits in the *Triticum aestivum* database and were identified as granule-bound starch synthase (GBSS I), two glutenins more precisely identified as glutenin subunits 1Dx7 and 1Dy9 and one glutenin protein which could not be classified in detail (Zörb et al., 2009) (chapter 3, tab. 1).

Starch has profound effects on breadmaking of wheat flour, since it is the most abundant component with about 70% of mature wheat grain (Dale and Housley, 1986). Starch is further essential during flour mixing process since it forms the characteristic starch-protein matrix. The matrix of starch and gluten proteins in wheat flour is able to retain gas during the fermentation phase of bread processing and is therefore crucial for loaf volume (Gan et al., 1995). Within the wheat grain, starch is organized in granules that further contain proteins that are called starch-granule-associated proteins. There are two groups of proteins associated with starch granules: First, gluten proteins (gliadins and glutenins) that remain absorbed at the surface of starch granules after extraction and second, the starch-granule-bound proteins that are distinct from gluten proteins and are tightly-bound to the surface or are integral components of the starch granule (Skerritt et al., 1990). To date, only two starch-granule bound proteins are named: the friabilins mainly consisting of puroindolines, and the SGBB I (Baldwin, 2001). The SGBB I is responsible for the synthesis of amylose in starch granules (Ring, 1995). Since the amylose/amylopectin ratio is crucial for starch properties like gelatinization, pasting and gelation, the SGBB I has profound effects on baking quality (Zeng et al., 1997). To conclude, the SGBB I is one protein that was changed in both cultivars after late S fertilization and therefore is able to explain the enhanced baking quality.

Three glutenin subunits were further identified to be increasingly synthesized after late S fertilization, whereof two subunits could more precisely be identified as HMW-GS 1Bx7 and 1By9 (Zörb et al., 2009) (chapter 3, tab. 1). HMW glutenin subunits are already highly investigated concerning its relation to baking quality (Payne, 1987; Seilmeier et al., 1991; Wieser and Zimmermann, 2000). Gupta et al. (1991) found glutenin concentration in wheat flour to correlate better with baking quality than flour protein concentration. However, glutenin concentration was similar in both treatments, high and late S fertilization, and therefore not quantity but composition of glutenins must be responsible for the increased loaf volume. In former investigations, single glutenins like 1Ax2\*, 1Dx5, 1By9 and 1Dy10 were found to be positively related to baking parameters (Payne et al., 1987; Weegels et al., 1996). Later, combinations of alleles (1Dx5 + 1Dy10 or 1Dx2 + 1Dy12) were used to explain baking quality (Branlard and Dardevet, 1985; Seilmeier et al., 1991), since the combination showed better correlation with breadmaking than single subunits. For example the combination of

1Dx5+1Dy10 is associated with high breadmaking quality whereas the combination 1Dx2+1Dy12 is not (Gupta et al., 1994).

Experimental data in chapter 3 show that HMW-GS were changed after late S fertilization. This aspect was observed in two years in the cultivar Türkis (chapter 3, fig. 6 and chapter 4, fig. 2) and as well in the cultivar Batis. The late S fertilization appears to change glutenin proteins that are already revealed to enhance baking quality characteristics of wheat.

The combination 1Bx7+1By9 is related to good baking properties (Seilmeier et al., 1991), and so far is a second factor that is able to explain the increased loaf volume after late S fertilization. However, it is difficult to distinguish between changes in protein composition as affected by fertilizer treatment or environmental conditions such as temperature regimens and precipitation during grain development. Independent from weather conditions, the identified proteins were as well found in the cultivar Batis and furthermore in the cultivar Türkis in year 2009 (chapter 4, fig. 2). So far, these proteins provide potential to explain a considerable part of the enhanced baking quality.

## **6.2. Influence of sulfur fertilization on storage protein composition during grain development**

Protein composition in mature grain was changed when comparing high S with late S fertilization. Secondly, it had to be clarified if a late S fertilization has an impact on protein composition as well as during early stages (chapter 4). Therefore, protein composition of milk ripe grain at early stages of grain development was analyzed.

Protein composition of the milk ripe grain was as well affected by late S fertilization (chapter 4). There were proteins mainly changed in the acid pH area. At milk ripeness HMW glutenins and LMW were not yet completely synthesized (Shewry et al., 2009), thus, changed proteins can be constituted to be albumins, globulins or gliadins (cf. chapter 3, fig. 6 with chapter 4, fig.2). Comparing changed proteins under milk ripeness with changed proteins at maturity, no proteins could be found that are changed in both developmental stages. So far, the impact of late S fertilization is available throughout

early and also late stages of grain development, whereas in early stages different proteins are affected than at late stages.

As Shewry et al. (2009) indicated by 1D-gel electrophoresis, the first abundance of proteins is detectable at 14 days post anthesis (dpa). During the first days of grain development albumins and globulins are synthesized (Gupta et al., 1996; Stone and Nicolas, 1996; Panozzo et al., 2001), whereas gliadins and glutenins accumulate at later stages. Shewry et al. (2009) further indicated that  $\omega$ -gliadins are starting to be synthesized in later stages of development and do not acquire more quantity after 30 dpa, whereas, HMW and LMW-GS are synthesized from 12 dpa until maturity. Late S fertilization is as a consequence affecting all Osborne fractions and not only glutenin proteins at maturity.

Tabé et al. (2002) and Shewry et al. (2001) further showed that as well storage protein genes are regulated by S-containing compounds and so far, N and S fertilization may directly influence the regulation of storage protein genes by enhancing their expression. Although no clear impact of single S-containing compounds on the expression of individual genes is yet known, late S fertilization may regulate different genes than high S fertilization. Shewry et al. (2009) further elucidated that also the expression of individual storage protein genes is changing during grain development. The HMW-GS 1Bx7 and 1Dy transcripts that were found to be changed after late S fertilization (chapter 2) are increasing during the first days of grain development (Shewry et al., 2009). It is interesting that the transcript of the 1Bx7 subunit is increasing until maturity whereas the 1Dy is expressed in almost constant amounts throughout the whole grain development (Shewry et al., 2009). Obviously, late S fertilization beneficially influences the expression of 1Bx7 transcripts and therefore higher amounts of this HMW-GS are available. This aspect may explain the increased loaf volume with late S fertilization.

In result, late S fertilization changes the whole wheat plant S metabolism throughout the whole time from S application until grain maturity.



### **6.3. Influence of sulfur fertilization on baking quality**

In the experiment conducted in 2007 late S fertilization resulted in higher loaf volume in both cultivars, Batis and Türkis (Zörb et al., 2009) (chapter 2, fig. 7). Loaf volume in Batis was enhanced by 3.6 mL and that of Türkis by 2.0 mL in a micro-scale baking test using 10 g wholemeal flour (Zörb et al., 2009). Unfortunately, these results could not be confirmed in the second year 2008 (data not shown). However, there are more investigations that already illustrated a positive effect of a late S fertilization on loaf volume (Seling et al., 2006). After late S fertilization loaf volume was increased by 117 mL 100 g flour<sup>-1</sup> in a rapid-mix-test (Seling et al., 2006).

To proof baking test data by an indirect method, the sedimentation test by Zeleny (1947) was conducted (data not shown). This method is highly correlating with loaf volume in baking tests. The principle of the Zeleny sedimentation test is based on swelling of gliadin and glutenin proteins within an ethanol-lactate-solution. In our experiments the sedimentation value increased continuously with S fertilization levels. Late S fertilization was found to show equal sedimentation values compared to high S fertilization, except one year where sedimentation values were higher. Since the sedimentation value correlated better with S fertilization than the micro-scale baking test, the question arose whether the micro-scale baking test with wholemeal flour is an adequate method to determine loaf volume. It is well known, that baking products result in lower loaf volume when prepared with wholemeal flour. This effect was as well confirmed by our investigations (chapter 5, fig. 2). However, does wholemeal flour also have adverse effects on loaf volume so that the illustration of S effects on loaf volume is impossible? The micro-scale baking test was the only baking test method available for the limited material resulting from pot experiments. In chapter 5 the suitability of different flour types in a micro-scale baking test is illustrated. The two cultivars, Batis and Türkis, have different baking potential resulting in higher loaf volume of the cultivar Türkis. With flour possessing low ash content (Type 550) the higher baking quality of cultivar Türkis was determined in both the rapid-mix-test and the micro-scale baking test (chapter 5, fig. 1 and 2). However, in a micro-scale baking test with wholemeal flour, Türkis lost more loaf volume than cultivar Batis and differences in baking potential could not be determined anymore. In this case, field experiments are necessary to obtain more grain material to conduct the rapid-mix-test with fine flour

(Type 550) and proof effects of late S fertilization with different cultivars and under different soil conditions.

#### **6.4. Advantages of a late sulfur fertilization in wheat production**

Sandy soil and high precipitation rates after first dressing or longer drought periods in spring inhibit optimal and adequate fertilization treatments for plant growth. These environmental conditions constrain farmers to react flexible with fertilization securing optimal plant growth, yield and quality.

A late N fertilization in comparison to an N fertilization at sowing and at tillering was in former times revealed to enhance protein concentration in wheat grain resulting in enhanced baking quality (Jahn-Deesbach and Weipert, 1964). Late applied N is accumulated mainly in ears, flag leaf and to a lower extent directly into the developing grain within a short time period prior to grain development (Simpson et al., 1983). Therefore, the question arose whether a late S fertilization in companion with a late N fertilization has further beneficial effects on baking quality, since S fertilization is known to fine-tune gluten proteins by increasing the synthesis of S-rich protein subunits (Wieser et al., 2004). It was not yet possible to show clearly an increased baking quality after a late S fertilization in field experiments. However, up to date a late S fertilization was as well not shown to be negative.

Bulk depositions of S in Europe are too low to cover S demands of crops (Schnug and von Franck, 1985; Zhao et al., 1995). Furthermore, there are soil conditions that do not contain enough amounts of plant available S to cover S demands. So far, soil conditions that can not cover S demands like sandy soils with low agricultural land grade that suffer from leaching may be appropriate for a late S fertilization. However, the impact of late S fertilization on different soil types was not yet reported and so far field experiments that show possible beneficial effects of a late S fertilization, maybe as well on good quality soils, are necessary.

A late S fertilization with respect to plant metabolism is beneficial, since S is applied when highest demand during ear and grain development is present. Since the demand is

high, sulfate is fast assimilated and incorporated into storage proteins. Therefore, negative feedback on sulfate transporter in roots might be reduced, since no lower-molecular S-containing compounds accumulate. Sulfur uptake is steadily acquired and may be more efficient than compared to an S application at early stages of growth.

As another aspect, weather conditions may influence late S fertilization adversely by causing high leaching of early applied sulfur applications. So far, our investigations revealed that a late S application is still incorporated into storage proteins and therefore is an alternative for at least sandy, easily leached soils. If during grain development weather conditions are dry, Tea et al. (2007) showed that a late foliar application of S is an alternative that increased dough strength, swelling and extensibility and therefore baking quality parameters.

In conclusion, these investigations reveal that a late S fertilization is an adequate fertilization technique to maintain high quality grain with increased levels of high potential glutenin subunits. Wheat plants have the ability to translocate late applied S into flag leaves, assimilating sulfate within a few days to glutathione that is transported towards the grain for maintaining S-rich storage protein synthesis. With late applied S it is possible to prevent S deficiency and compensate latent S deficiency appearing during early stages of plant growth.

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### 7. Conclusion

Baking quality of wheat is influenced by gluten proteins that are predominantly determined by the genetic background of the wheat cultivars. However, their quantity and quality can be changed by N and S fertilization. Late N fertilization is already known to enhance protein concentration in mature wheat grain and therefore benefits baking quality. Sulfur fertilization at sowing increases S-rich storage proteins such as  $\gamma$ -gliadins and low molecular weight (LMW)-glutenin subunits and further influence baking quality, since disulfid-bridges in storage proteins are primarily important for an adequate starch-gluten-network.

The objective of this thesis was to determine whether a late S fertilization concomitantly with a late N fertilization further benefits storage protein composition resulting in changed baking quality parameters. Pot experiments with two winter wheat cultivars, Batis and Türkis, differing in baking quality but not in protein concentration in wheat grain, were conducted. Increasing S fertilization levels with 0 g S, 0.1 g S, 0.2 g S at sowing and one late S fertilization level with 0.1 g S at sowing + 0.1 g S at ear emergence was applied.

When applying a late S fertilization, sulfate is predominantly transported to the flag leaf and assimilated to glutathione, instead of direct transport to the developing ear. In mature wheat grain a late S fertilization does not change protein or S concentrations but storage protein composition and thus protein quality. Changes in protein composition were found in both, mature and milk ripe grain. As a result late S fertilization influenced protein composition as well in early stages of development. By proteome analysis it was possible to identify starch-granule bound starch synthase (GBSS I) and high molecular weight (HMW)-glutenin subunits such as 1Dx7 and 1Dy9 to be synthesized in higher amounts after a late S fertilization than compared to high S fertilization at sowing (0.2 g S) in mature wheat grain. These results were shown for both cultivars, Batis and Türkis. The combination of HMW glutenin subunits 1Dx7+1Dy9 is known to be beneficial for baking quality.

Due to limited material resulting from pot experiments a standardized micro-scale baking test with wholemeal flour instead of fine flour was conducted for the first time.

A late S fertilization increased loaf volume in both cultivars in one year but not in a second year of experiments.

However, late S fertilization was never shown to influence protein composition and baking quality in a negative way and so far functions as an alternative to S fertilization at sowing. Thus, late S fertilization is a fertilization technique that prevents S deficiency in late stages of wheat growth and further enables equal amounts of S and protein compared to an S application only at sowing with an increased protein quality.



## 8. Zusammenfassung

Die Backqualität von Weizen wird zu einem erheblichen Anteil durch eine N- und S-Düngung bestimmt. Unabhängig von der genetischen Prädisposition einzelner Sorten kann eine N- und S-Düngung die Speicherproteinkonzentration und -qualität positiv beeinflussen. Eine N-Spätdüngung zum Ährenschieben erhöht beispielsweise die Proteinkonzentration im Korn und damit die Backqualität von Weizen. Es ist bereits bekannt, dass eine S-Düngung zur Aussaat den Anteil S-haltiger Speicherproteine wie  $\gamma$ -Gliadine und Low Molecular Weight (LMW)-Gluteline im reifen Weizenkorn erhöht. Somit werden Disulfidbrückenbindungen zwischen den Speicherproteinen ausgebildet, welche ein für Backprozesse adäquates Gluten-Stärke-Netzwerk ermöglichen.

Das Ziel dieser Arbeit war es, aufzuklären, ob es möglich ist mit einer S-Spätdüngung, die gleichzeitig mit einer N-Spätdüngung gegeben wird, die Speicherproteinzusammensetzung und damit die Backqualität zusätzlich positiv zu beeinflussen. Hierzu wurden Gefäßversuche mit zwei Winterweizensorten, Batis und Türkis angesetzt, welche sich in ihrer Backqualität, nämlich dem Gebäckvolumen, aber nicht in der Quantität der Proteine im Korn unterscheiden. Die beiden Sorten wurden mit unterschiedlichen S-Düngungsstufen gedüngt: 0 g S; 0,1 g S und 0,2 g S zur Aussaat und einer S-Spätdüngungsvariante mit 0,1 g S zur Aussaat und 0,1 g S zum Ährenschieben.

In Bezug auf die Verteilung von S-haltigen Komponenten in verschiedenen Weizenorganen konnte in dieser Arbeit festgestellt werden, dass Sulfat nach einer S-Spätdüngung zunächst in das Fahnenblatt aufgenommen und zu Glutathion assimiliert wird, statt direkt in die Ähre verlagert zu werden. Im Korn bewirkte eine S-Spätdüngung im Vergleich zu einer S-Düngung zur Aussaat zwar keine Änderung der Protein- und S-Konzentration, jedoch war die Proteinzusammensetzung verändert. Diese Veränderung war sowohl im reifen Korn, als auch in frühen Entwicklungsstadien, nämlich während der Milchreife nachzuweisen. Mit Hilfe der Proteomanalyse wurde zum Zeitpunkt der Vollreife die Stärkesynthase und die High Molecular Weight (HMW)-Gluteninuntereinheiten 1Dx7 und 1Dy9 als verstärkt synthetisierte Proteine identifiziert. Aus der Literatur ist bekannt, dass eben diese HMW-Untereinheiten die Backqualität positiv beeinflussen. Aufgrund der geringen Mehlausbeute aus

Gefäßversuchen, wurde der Mikrobackversuch erstmalig standardisiert auf die Verwendung von Vollkornmehl angepasst. Durch eine S-Spätdüngung war das Gebäckvolumen in beiden Sorten in einem von zwei Versuchsjahren erhöht.

Zusätzlich wurde gezeigt, dass eine S-Spätdüngung die Proteinkonzentration und -zusammensetzung nicht negativ beeinflusst, sondern gleich hohe Protein- und S-Konzentrationen wie eine alleinige Düngung zur Aussaat gewährleistet. Eine S-Spätdüngung kann somit auch noch in späten Entwicklungsstadien der Pflanze einem S-Mangel vorbeugen und diesen sogar kompensieren.

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