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

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Increasing prices for wheat products and fertilizers, as well as reduced sulfur (S) contributions from the atmosphere, call for an improvement of product quality and agricultural management. To detect the impact of a time-dependent S fertilization, the quantitative protein composition and the baking quality of two different wheat cultivars, Batis and Türkis, were evaluated. The glutathione concentration in grains serves as a reliable marker of the need for added S fertilizer. The quantitation of gliadins and glutenin subunits by reversed-phase high-performance liquid chromatography confirmed that S-rich proteins significantly increased with S fertilization, whereas the S-poor proteins significantly decreased. Proteome analysis by means of high-resolution protein profiles detected 55 and 37 proteins from Batis and Türkis changed by late S fertilization. A microscale baking test using wholemeal flour was implemented for the evaluation of baking quality, and late S fertilization was found to improve the composition of gluten proteins and baking quality.

KEYWORDS: Wheat; baking quality; N and S fertilization; proteomics; gliadin; glutenin

INTRODUCTION

Current findings showing a reduction of sulfur (S) entries from atmospheric deposition as well as increasing prices for agricultural inputs warrant improvement of agricultural S fertilization management. Because industrial emission of S has decreased, wheat deficiency symptoms have increased. Although wheat only requires modest amounts of S for optimum growth, deficiency of S is known to reduce yield and baking quality of wheat (*Triticum aestivum*) flour (*1*−*4*). S deficiency has a profound effect on the composition of gluten proteins in the wheat grain with increased synthesis of S-poor proteins such as ω-gliadins and high molecular weight subunits of glutenin (HMW-GS) at the expense of S-rich proteins such as γ-gliadins and low molecular weight subunits of glutenin (LMW-GS) (*1*, *5*−*7*). Grains assimilate 50% of S during anthesis and maturity (*3*), which implies that S availability should be maintained throughout the whole period of growth. Late application of S before head emergence may become a relevant strategy for S fertilization. A time-dependent application of S is assumed to be important because the S demand is high during grain filling and S is susceptible to leaching, especially in sandy soils. Therefore, the effect of a late S fertilization was introduced in this experiment. Glutathione (γ-Glu-Cys-Gly; GSH) is usually present at high concentrations in most living cells next to sulfate and is a major reservoir of nonprotein-reduced S (*8*). The pools of S and the transport of GSH to the grain during grain fill may be affected by different S fertilization timings. Because of GSH's unique redox and nucleophilic properties, GSH additionally prevents oxidative stress (*9*), and a balanced GSH concentration in grain is associated with a beneficial effect on baking quality (*10*). Therefore, measurement of the GSH concentration in the grain would be a suitable tool to obtain information about the S nutrition status of the plant according to the S fertilization rate.

Along with S nutrition, nitrogen (N) nutrition of wheat influences growth and yield together with biochemical parameters, such as the concentration of storage proteins and baking quality (*11*−*14*). However, the current practice of applying large amounts of N fertilizers to cereal crops without considering S requirements is becoming a concern for crop and grain quality (*15*).

The aim of this study was to detect the effect of late S fertilization on the pattern of storage (gluten) proteins such as gliadins and glutenins and their relationship to the baking quality. These results will contribute to a better understanding of S nutrition of wheat and the improvement of baking quality by adjusting S fertilizer timing and S fertilizer rate.

MATERIALS AND METHODS

Plant Cultivation. Winter wheat cultivars Batis and Türkis were grown under defined conditions in a greenhouse. Both were chosen because, according to the German Federal Office of

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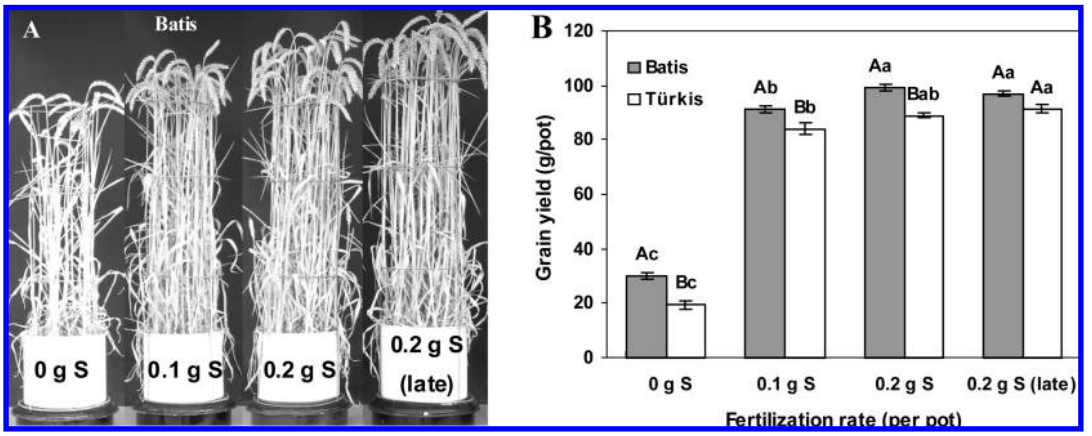

Figure 1. (**A**) Wheat plants (cv. Batis) in pot experiments with a soil mixture of 2/3 quartz sand and 1/3 soil. S fertilization rates represented 0, 0.1, 0.2, and 0.1 g plus an additional 0.1 g of late S fertilization per pot. For details, see the Materials and Methods. (**B**) Effect of different S fertilization rates on the grain yield (g/pot). Different letters represent significant differences of mean values. Error bars represent ±standard errors of five independent pot replicates. Statistical significance ($p \leq 0.05$) is indicated by small letters for the S rates and capitals for the cultivars.

Plant Varieties (*16*), these cultivars are classified within the same baking quality class (A) and show similar quality parameters in terms of crude protein concentration, bread volumes, and rheological parameters. Pots were filled with identical soil in Mitscherlich pots containing 6 L of soil (1/3 loam and 2/3 quartz sand) and increasing amounts of $MgSO_4$. Three different S fertilization rates were applied by adding 0 (none), 0.1 (moderate), and 0.2 g (high) S/pot before sowing (EC 01). Additionally, a late S fertilization with 0.1 g S/pot before sowing and 0.1 g S/pot at head development was applied (EC 43, **Figure 1A**). Each wheat cultivar was replicated five times. N was applied at a single rate (1.0 g of N, NH_4NO_3) at three different times (EC 01, 11, and 43) during the growing season (*17*). Phosphorus (5.0 g $CaHPO_4$/pot), K (3.4 g KCl/pot), Mg (1.0 g $MgCO_3$/pot), and the minor elements were applied at recommended rates for optimal growth.

Yield and N, S, and GSH Concentrations. Wheat grains were milled in a Titan laminated mill using a 500 μm sieve (Retsch, Haan, Germany). The wheat flour was further ground to a fine homogeneous wholemeal powder using a mortar. S and N concentrations were determined after a Dumas combustion method using a CNS elemental analyzer (Vario MAX CNS, Hanau, Germany). The protein concentration was then calculated by multiplying the N concentration by a factor of 5.7 (*18*). GSH concentrations were analyzed according to Langenkämper et al. (*19*) using an enzymatic method.

Microscale Baking Test. The microscale baking test with wholemeal flour was performed according to a procedure described in detail by Koehler and Grosch (*20*). Each 10 g of wholemeal flour was adjusted to 14% humidity. The yeast was checked for activity by using wheat flour (cv. Tommi) with an ash content of 0.55% as a standard. After it was kneaded with a microfarinograph (Brabender, Duisburg, Germany) to a consistency of 500 BU (Brabender units), the dough was rounded and incubated in a fermentation chamber. After an incubation time of 20 min, the rolls were baked at 230 °C for 10 min. Each sample was replicated two or three times according to the amount of material available. The volume of the microbreads was determined by the water displacement of paraffin-coated breads.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) Analysis. The extraction of protein fractions and their quantitation by RP-HPLC were carried out according to Wieser et al. (*21*). Osborne protein fractions of wholemeal flour (100 mg) were extracted stepwise: (i) albumins/globulins, 0.4 M NaCl, 0.067 M Na_2HPO_4/KH_2PO_4, pH 7.6 (2 × 1 mL) at 20 °C; (ii) gliadins, 60% ethanol (3 × 0.5 mL) at 20 °C; and (iii) glutenin subunits, 50% (v/v) 1-propanol, 0.05 M Tris-HCl (pH 7.5), 2 M urea, 1% dithiothreitol (DTT), at 60 °C. Gliadins and glutenin subunits were separated and quantified by RP-HPLC on C_8 silica gel at 50 °C using a solvent gradient with increasing acetonitrile concentration in the presence of 0.1% (v/v) trifluoroacetic acid. The flow rate was 1 mL, and the detection wavelength was 210 nm. At least two determinations were performed for each analytical assay.

Protein Extraction for Two-Dimensional Gel Electrophoresis (2D-GE). The wholemeal flour (see Yield and N, S, and GSH Concentrations) was further ground to a fine homogeneous powder under liquid N using a mortar. Proteins were extracted from 100 mg of wholemeal flour using a modified DTT−trichloroacetic acid (TCA)−acetone precipitation method [for further details, refer to Zörb et al. (*22*)]. To inhibit protease activity, strong denaturants (urea and TCA) and a protease inhibitor cocktail (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) were incorporated into the extraction solution. Wheat flour protein concentrations of the samples were determined using a 2D Quant protein quantification kit (GE Healthcare, Munich, Germany).

Isoelectric Focusing (IEF) and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). To separate grain proteins, 2D-GE was performed using the protocols of O'Farrell (*23*). Commercially purchased immobilized pH gradient (IPG) strips (11 cm, pH 3−10, linear, GE Healthcare, United States) were used for IEF. During 10 h of rehydration, IPG strips were loaded with 300 μg of protein. IEF was carried out in an Ettan IPGphor focusing system (GE Healthcare) at the following conditions: 100 V for 2 h, 500 V for 1 h, 1000 V for 2 h, and 8000 V for 2 h. The system temperature was 20 °C, and the current was 45 μA per strip. For the second dimension, a 16 cm × 16 cm vertical gel electrophoresis chamber (Hoefer, Munich, Germany) was used. The SDS-PAGE gels had a concentration of 12.5% (v/v) acrylamide. After IEF, the IPG strip and molecular mass standards in the range from 10000 to 150000 (Sigma, Germany) were applied onto the gel surface, which was subsequently sealed with 1% (w/v) agarose containing 0.001% (w/v) bromophenol blue. Running conditions were 25 °C at a constant current of 45 mA per gel. Electrophoresis was stopped, when the bromophenol blue reached the gel margin. The gels were fixed with 50% ethanol and 10% acetic acid, and protein staining was done at 80 °C with Coomassie R350 (GE Healthcare).

Image Analyses. Gels were scanned in the trans-illumination mode (HP Scan-Jet 4890, United States; 300 dpi and 16 bits per pixel). Computer-assisted 2D-GE analysis was performed with Delta2D 3.4 (Decodon GmbH, Greifswald, Germany). Gels were warped using a group warping strategy to obtain an average fusion gel. A virtual master fusion gel was created by the software using every matched protein spot from all gels. The

master fusion gel was then used to delete artifacts and specks on individual gels before further processing. Subsequently, the protein spot intensity was determined for all individual gels. Average relative protein spot intensities were calculated to compare high S and late S fertilization treatments.

Protein Identification. Mass spectrometry (MS) and protein identification were performed at the Center for Molecular Medicine University of Cologne (Germany) using a Proteineer spot picking robot system (Bruker, Bremen, Germany) with subsequent automatic acquisition of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer on a Bruker Reflex IV MALDI-TOF MS controlled by FlexControl 1.3 software (Bruker). Database searches were performed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) number release 2006/1017 using MASCOT 1.9. The molecular mass of the proteins was calculated on the basis of a standard 10000 MM ladder using 2D software. The pI of the protein spots was calculated from their position on the IEF strips as mentioned by the manufacturer's specification (GE Healthcare).

Data, Replication, and Statistics. For proteomics, the reliability of the results was ensured by the following measures: (i) Two technical 2D-GE gel replications from grains of high S and late S fertilization that were used to create an average 2D-GE gel. (ii) The average mean value and standard errors of 2D-GE gels that were calculated from the three independent biological pot replicates. (iii) Student *t* tests were performed to evaluate the reproducibility of the spots over the corresponding biological replicates (high S and late S fertilization). Spots were disregarded when the *t* test value was below 90%. (iv) A speckle filter with an intensity limit of <0.02 was applied. (v) Differences in protein spot ratios of high S and late S fertilization were disregarded, unless different by a factor of 2 (changing factor of double or half).

All statistical analysis was carried out using SAS (SAS Institute Inc., Cary, NC, Release 9, 2001). Comparisons of means with respect to the influence of S supply and the two cultivars Batis and Türkis (genotype) were carried out using the GLM procedure considering a two-factorial, orthogonal, fully randomized design. The homogeneity of variances and normal distribution were evaluated by plotting studentized residues over predicted values (means) and by the Shapiro−Wilk test, respectively. Where appropriate, data were either square-root or log transformed to maintain homogeneity of variance. To maintain an experiment wise α of $p < 0.05$, multiple *t* tests were adjusted according to Bonferroni-Holm (*24*). Statistical significance is indicated by small letters for the S rates and capitals for the cultivars.

RESULTS

Grain Yield. Generally, S fertilization together with appropriate N supply increased grain yield 3-fold as compared to nonfertilized wheat (S deficiency, **Figure 1A**,**B**). The similar S fertilization effect was previously reported by other authors using pot experiments as well as field experiments (*3*, *7*, *25*, *26*). In our experiment, grain yield was significantly increased at high S fertilization rate (0.2 g/pot) with Batis as compared to the moderate fertilization rate (0.1 g/pot) (**Figure 1A**,**B**). In comparison to the high S fertilization rate, the newly introduced late S fertilization rate (both 0.2 g/pot) showed no significant effect on yield but a tendency for a higher yield with late S fertilization in Türkis. When comparing cultivars, Batis had a higher grain yield than Türkis except the late S fertilization rate.

Wheat Grain S and GSH Concentration. The S concentrations of both cultivars with and without S fertilization were in the range of 0.8−1.6 mg/g (**Figure 2A**). Ratios of N to S higher than 17:1 indicate S deficiency (*27*). In our experimental design, S deficiency was achieved when wheat was not fertilized and had N/S ratios of 32.8 and 32.4 for Batis and Türkis, respectively, while N/S ratios of 22.1 and 22.5 were achieved for Batis and Türkis at the moderate S fertilization rate (**Figure 2B**).

The GSH concentration was below 80 μg/g flour in both, at the low rate and nonfertilized wheat grains (**Figure 3**). The GSH concentrations at both high S fertilization rates were above 200 μg/g wholemeal flour. The differences between the high and the late S fertilization treatments were not significantly different, but a tendency was indicated. Thus, a clear correlation between the S fertilization rate and the GSH concentration in Batis and Türkis was detected.

Wheat Grain Protein Yield and Protein Profile (HPLC and Proteomics). Grain total protein content is a widely accepted criterion to assess the baking quality of wheat flour. In this study, this parameter was characterized by determining the protein yield per pot. To avoid a lack of N for optimal plant growth, a high N fertilization rate according to practice was used in all variants. The protein yield of both cultivars in non-S fertilized wheat was below 5 g/pot, whereas the protein yield under moderate and high S fertilization was more than double as high with up to 12 g/pot (**Figure 4**). No significant differences between the high S and the late S fertilization in terms of protein yield of both cultivars were detected.

The grain quality analyzed by means of classical Osborne fractionation and RP-HPLC demonstrates the impact of storage protein types. Moreover, a high definition 2D-GE was used to show differences in the protein pattern of soluble proteins up to the characterization of single proteins. In general, the results of the RP-HPLC were comparable to previous findings (*6*) but provided more details. In **Figure 5**, the results of the quantitative analysis of gluten protein types by RP-HPLC are illustrated. With the exception of grains at the 0 g of S rate, the concentration of the total gliadin fraction is nearly double than the total glutenin fraction (**Figure 5A**,**B**). The total gliadin fraction was not notably changed by the S treatments, whereas the total glutenin fraction was significantly lower at the 0 g of S rate. No clear difference between the gliadin and the glutenin subunit concentrations under high S fertilization rates and late S fertilization treatments was detected. A more detailed analysis of the different gliadin types showed that the γ-gliadins significantly increased with higher S fertilization, whereas the ω1,2- and ω5-gliadins significantly decreased with increasing S fertilization in both cultivars (**Figure 5C**,**D**). A more detailed analysis of the glutenin subunits showed that the LMW-GS significantly increased with higher S fertilization in both cultivars (**Figure 5E**,**F** and Figure 8 in Supporting Information). Likewise, the HMW-GS were lowest with no S fertilization rate in Batis and with no and late S fertilization rate in Türkis (**Figure 5E**,**F**). The glutenin-bound ω-gliadins (ωb) were low as compared to the HMW- and LMW-GS. However, the ωb-fraction followed the same pattern as observed for HMW-GS. With no S fertilization, the ωb-fraction was as high as with high S fertilization. Notable differences among the cultivars were present especially in the gliadin fraction, where the cultivar Türkis showed higher α-gliadin concentrations than the cultivar Batis.

The proteome analysis using 2D gels provided access to the relative concentration changes of single proteins and to identify the changed proteins. The HPLC analysis of gliadins and glutenin subunits (**Figure 5A**,**B**) revealed some differences between high and late S fertilization treatments of storage protein subunits. Therefore, we focused the proteo-

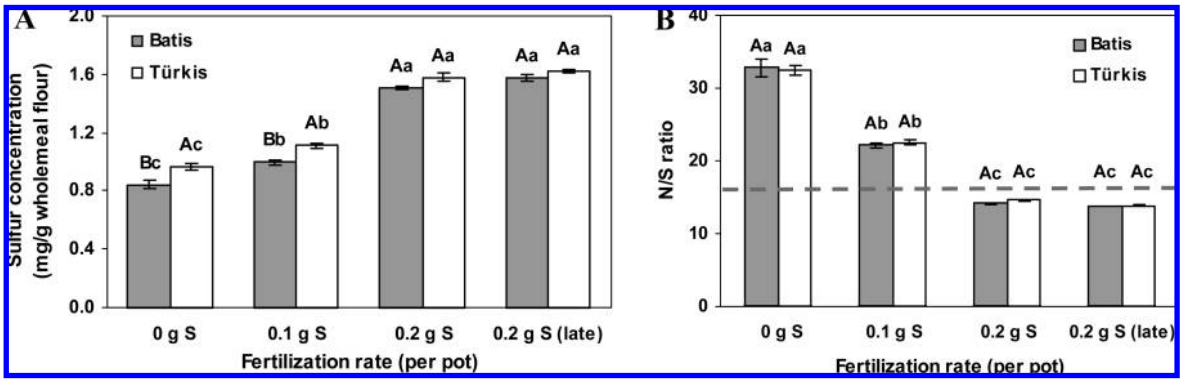

Figure 2. (**A**) S concentrations (mg/g wholemeal flour) in wheat grains (cv. Batis and cv. Türkis) with different S fertilization treatments (0, 0.1, 0.2, and 0.1 g plus additional 0.1 g of late S fertilization). Different letters represent significant differences of mean values. Error bars represent ±standard errors of five independent pot replicates. Statistical significance ($p \leq 0.05$) is indicated by small letters for the S rates and capitals for the cultivars. (**B**) Effect of different S fertilization rates on the N/S ratio of two wheat cultivars. The N/S ratio above the dotted line indicates S deficiency. Different letters represent significant differences of mean values. Error bars represent ±standard errors of five independent pot replicates. Statistical significance ($p \leq 0.05$) is indicated by small letters for the S rates and capitals for the cultivars.

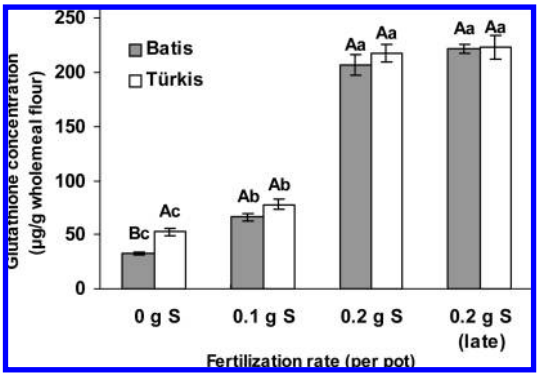

Figure 3. Effect of different S fertilization rates on the GSH concentration (μg/g wholemeal flour) of two wheat cultivars. Different letters represent significant differences of mean values. Error bars represent ±standard errors of five independent pot replicates. Statistical significance ($p \leq 0.05$) is indicated by small letters for the S rates and capitals for the cultivars.

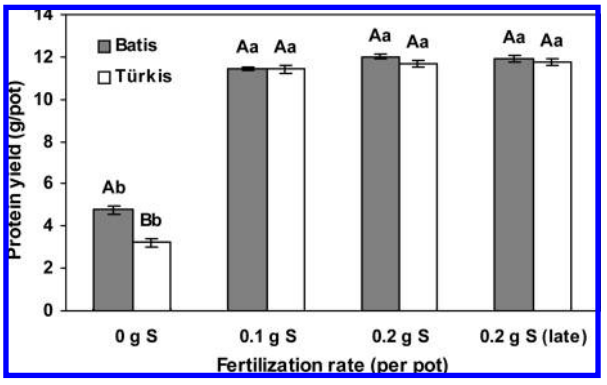

Figure 4. Effect of different S fertilization rates on the protein yield (g/pot wholemeal flour) of two wheat cultivars. Different letters represent significant differences of mean values. Error bars represent ±standard errors of five independent pot replicates. Statistical significance ($p \leq 0.05$) is indicated by small letters for the S rates and capitals for the cultivars.

mics analysis on both S regimes to characterize single proteins, which were changed. The proteome analysis of the wheat grains from the high and late S fertilization revealed a total of 388 and 372 proteins from Batis and Türkis, respectively. A 2D gel specimen from late S fertilization of Batis and Türkis is depicted in **Figure 6A**. The resolution of the 2D gels was in the same range as other research found in the literature (*28*, *29*). In total, 132 of these spots on the 2D gels were identified by MALDI-TOF analysis and a subsequent database search. Only proteins that differed by a factor of 2 or more for the high S and late S fertilization treatments were taken into further account. A set of 55 and 37 proteins from Batis and Türkis, respectively, differed statistically ($p \leq 0.05$) between high S and late S fertilization (**Figure 6A**). Most of these proteins, 45 from Batis and 28 from Türkis, showed higher concentrations after the late S fertilization, which represented 82 and 76% of the total changed proteins in Batis and Türkis, respectively (**Figure 6A**). In detail, the starch synthase concentration increased by late S fertilization in both cultivars, and the HMW-GS 1Bx7 as well as the HMW-GS 1By9 and one unspecified HMW-GS also notably increased in the cultivar Türkis (**Figure 6B** and **Table 1**).

Baking Quality. Finally, baking quality of wheat grain includes at least besides protein quantity the storage protein composition (*7*) and concentrations of low molecular mass thiols such as GSH (*10*). For the support and the extension of the biochemical quality parameters, it is necessary to apply a baking test to show the direct effects of the different S regimes. However, pot experiments only offer a small amount of material especially from S-deficient plants. Therefore, a microscale baking test with wholemeal flour had to be introduced. For this purpose, the microscale baking test described by Koehler and Grosch (*20*) was used and modified for wholemeal grain. **Figure 7A** shows images of microbreads under all S regimes of the cultivar Batis. In comparison to the high S-fertilized wheat, the bread volume of the nonfertilized wheat averaged 20 and 30% lower for Batis and Türkis, respectively (**Figure 7B**). The moderate S fertilization rate exhibits no significant difference in bread volume as compared to the high S fertilization rate. In spite of similar S concentrations (**Figure 2A**) and protein yields (**Figure 4**), a higher bread volume was detected comparing high and late S fertilization (**Figure 7**). The late S fertilization treatment resulted in a 10 (significant) and 6% (not significant) increased bread volume for Batis and Türkis, respectively (**Figure 7B**).

DISCUSSION

Because of limited pollution and reduced acid rain, visible S deficiency symptoms in winter wheat are present in several areas when no S fertilization is applied (*30*). As wheat fertilizer

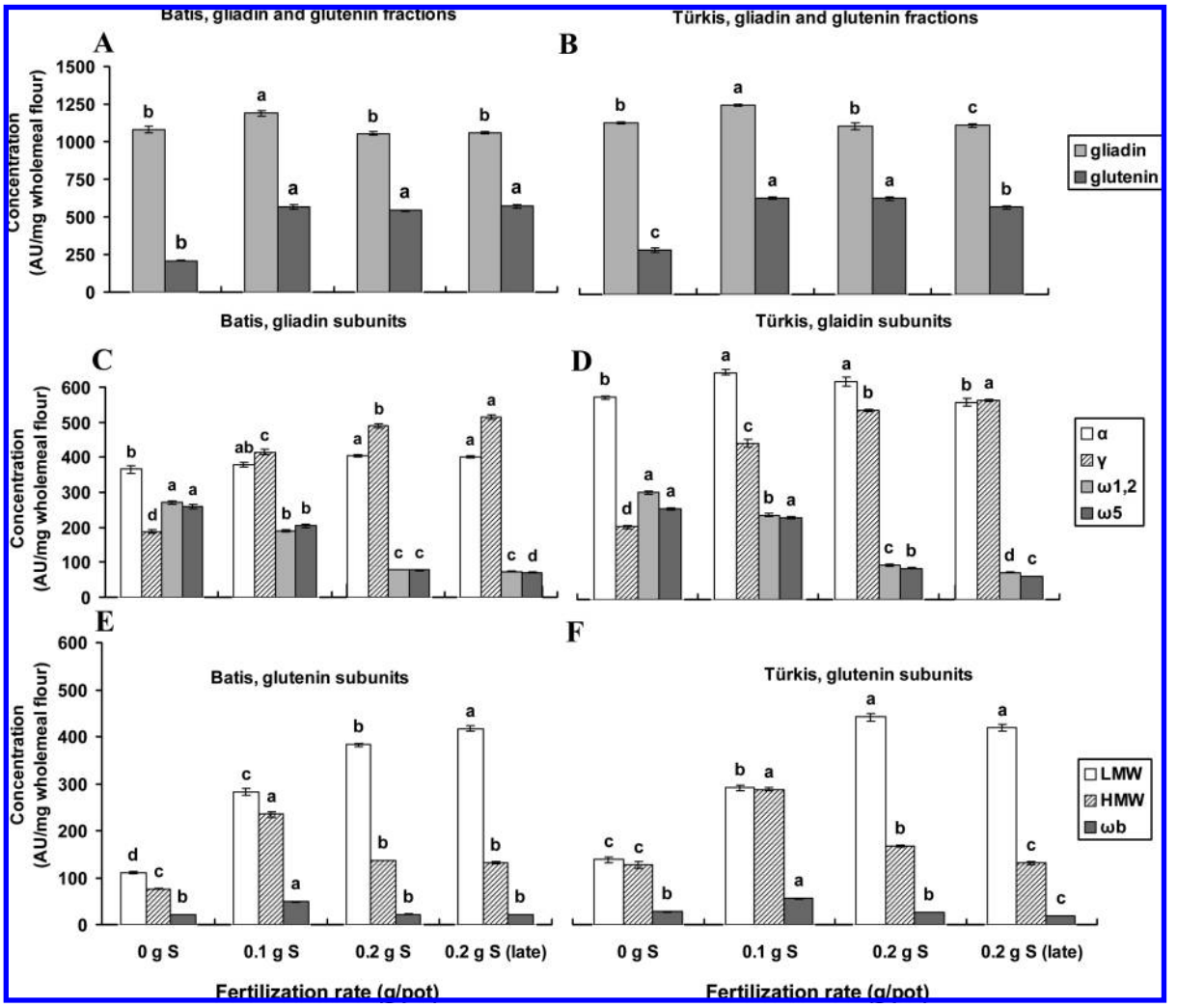

Figure 5. Effect of different S fertilization rates on the concentration of gluten proteins (AU/mg flour) in wholemeal flour of Batis and Türkis analyzed by RP-HPLC. AU, relative absorbance units. (**A** and **B**) Total Osborne fractions of gliadins and glutenins, (**C** and **D**) gliadin types, and (**E** and **F**) glutenin subunits. Statistical significance ($p \leq 0.05$) is indicated by small letters for the S rates.

inputs continue to rise around the world, there is a need for crop fertilizer management innovations. Furthermore, proper S fertilization is needed to obtain a good baking quality (*3*). It has long been known that during N deficiency, sulfate assimilation is reduced and that the capacity to reduce nitrate is diminished in plants with S deficiencies (*31*). Therefore, in this study, a high N fertilization rate plus a late N fertilization increment according to agricultural practice was used. Besides a possible interaction from a low N supply, the S concentration in the grain is markedly influenced by the S supply to the plant (*27*). The form of S given to a field crop depends on factors such as availability, mobilization, mineralization, and uptake mechanisms of the plant root from the soil solution. For instance, a foliar application of 10 kg S/ha (in elemental S-form) at the grain milky ripe stage of wheat kernels was reported to have little or no effect on grain S concentration in 2 years in Great Britain, but ammonium-sulfate treatments led to higher S concentrations than elemental S and an increase in grain S concentration.

Yield, Protein, S, and GSH Concentrations, and N/S Ratio Baking Quality. Greenhouse and field studies confirmed that the effect of S deficiency is primarily on the number of grains per head (*3*). We also confirmed a grain yield decrease of 70% under S deficiency in the pot experiment. Additionally, our results indicated significant effects of S fertilization on the S concentration, the protein yield, and the N/S ratio of wheat grains (**Figures 2B** and **4**). It was reported that increasing rates of S fertilization had negligible effects on the protein content unless there is a deficiency situation (*7*), which agrees with our findings (**Figure 4**). Comparing high S fertilization rates with late S fertilization, no clear difference in protein yield and S concentration could be determined (**Figures 4** and **2A**). Studies on redistribution of S in wheat during generative growth (*32*) showed that wheat plants, which acquired exogenous S, did not accumulate S in vegetative tissues but did supply the grain. In comparative studies on wheat, plants without S supply during postanthesis obtained S from soluble S sources in the leaves. An imbalance of N/S higher than 17:1 indicating S deficiency resulted in accumulation of nonprotein compounds like amides that negatively affect baking quality (*13*). Because of the high N/S ratio of the non-S-fertilized and the moderate S fertilization rate (**Figure 2B**), S deficiency was indicated and a decrease in baking quality might be expected. However, only the non-S-fertilized grains revealed a significant reduction of the bread volume (20% less), coupled with negative rheological properties that were observed. Interestingly, the moderate S fertilization rate did not show a significant effect with bread volume (**Figure 7**). However, moderate S treatments showed a reduced grain dry weight,

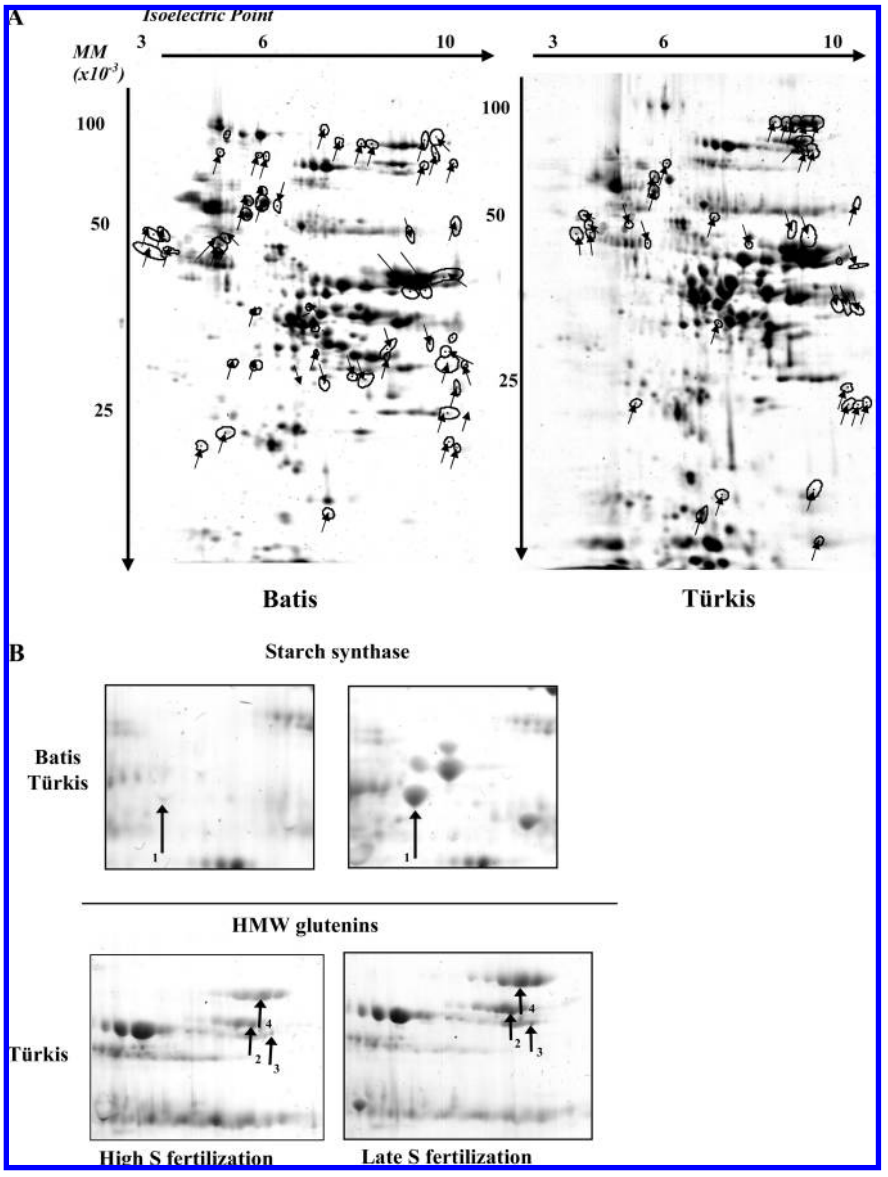

Figure 6. (**A**) Effect of late S fertilization (0.1 g of S plus 0.1 g of S) in comparison to high S fertilization (0.2 g of S) on the wheat grain proteome. Images of 2D gels of wheat grain proteins from two cultivars (cv. Batis and cv. Türkis). MM, molecular mass. Direction of arrow upward, increased protein concentration (spot intensity) by late S fertilization; direction of arrow downward, decreased protein concentration. (**B**) Section parts of **A** with four identified proteins: starch synthase, HMW glutenin subunit 1Bx7 and subunit 1By9, and one unspecified HMW glutenin subunit.

Table 1. X-Fold Protein Change of Late S Fertilization Range as Compared to High S Fertilization, Analyzed by 2D-GE and Identified by MALDI-TOF[a]

spot no.	X-fold change (high S vs late S)	identification	accession no.
1	2.7	starch synthase (GBSSI)	gil4760580
2	2.2	HMW subunit 1by9	gil62548385
3	2.4	HMW subunit	gil29569237
4	3.1	HMW subunit 1Bx7	gil109452233

[a] Spot numbers are according to **Figure 6B**. Accession numbers are according to a database (http://www.ncbi.nlm.nih.gov).

which is significant for Batis (**Figure 1**) and a significant lower S concentration in grains of Batis and Türkis (**Figure 2A**). However, protein yield was not affected by high S fertilization. By comparing high S fertilization rates with late S fertilization, an improvement of the baking quality was indicated but was not significant for the cultivar Türkis.

GSH is usually present at high concentrations in most living cells, being the major reservoir of nonprotein reduced S. Cysteine, the initial product of sulfate assimilation in plants, and its immediate metabolite GSH are both cellular constituents (*31*, *33*, *34*). Sulfate assimilation is an essential pathway, a source of reduced S for various cellular processes and for the synthesis of GSH, a major factor in plant stress defense. As S assimilation has a huge impact on the formation of GSH, a correlation between S fertilization, GSH formation, and baking quality was already examined by Reinbold et al. (*10*). Our results showed a distinct relationship between S fertilization and baking quality (**Figure 7**). The higher the S fertilization rates were, the higher the GSH concentration within the grain was. Therefore, we conclude that the GSH concentration in the grain may serve as a reliable marker for the S availability and the S supply of wheat. The time-dependent S fertilization in comparison between high and late S fertilization rates only showed slightly higher but no significant concentration increase of GSH in the late S-fertilized grains. It was recently published that GSH and cysteine both influence the rheological properties of dough and the baking performance (*10*).

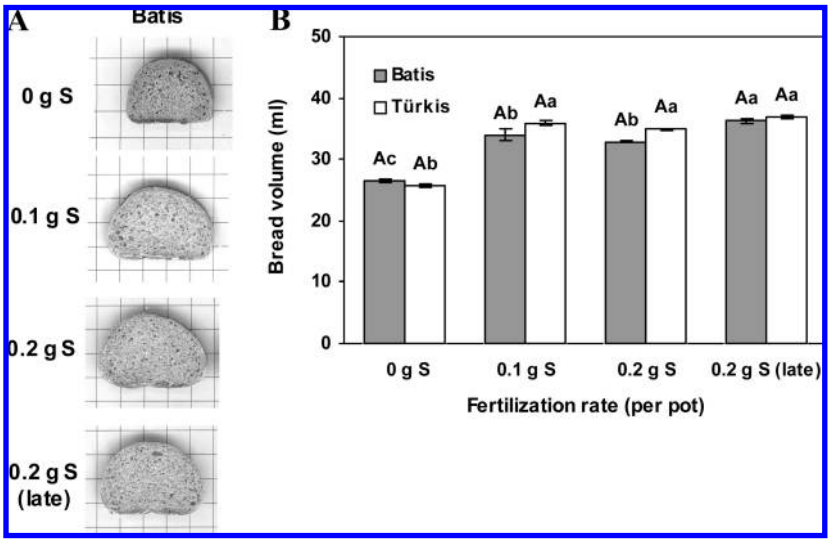

Figure 7. Effect of different S fertilization rates on the baking quality of two wheat cultivars as measured by a microscale baking test using 10 g of wholemeal flour. (**A**) Images of micro bread slices of the cultivar Batis at a comparable scale. (**B**) Histograms of bread volumes; different letters represent significant differences of the mean values. Error bars represent ±standard errors of five independent pot replicates. Statistical significance ($p \leq 0.05$) is indicated by small letters for the S rates and capitals for the cultivars.

Gliadin and Glutenin Storage Proteins and Effect of Late S Fertilization. It is well-known that the timing of a late N application at head emergence enhances the crude protein concentration by the increase of storage protein synthesis (*14*). Likewise, late S application is assumed to be a factor to improve the grain quality. The majority of S in mature wheat grains is present in proteins like B and C type LMW-GS, α- and γ-gliadins (*35*). Different supplies of S during wheat growth are known to influence the quantitative composition of gluten proteins (gliadins and glutenins) in flour. Former results (*7*) demonstrated that the amount of total gluten proteins as well as of crude protein content of flour was barely influenced. However, amounts and proportions of single protein types were strongly affected by different S fertilization strategies. With S fertilization, S-poor proteins like HMW-GS and ω-gliadins decrease, while S-rich proteins such as LMW-GS and γ-gliadins increase, which results in a shift of the gliadin/glutenin ratio (*5*, *36*−*38*). In general, our results confirmed these findings (**Figure 5**), but there were some unexpected effects. The amount of total gliadin fractions (**Figure 5A**,**B**) was only slightly changed with higher S fertilization regimes, but the total glutenin fractions showed the lowest concentrations with no S fertilization. We confirmed only a minute influence on total gliadin and glutenin concentrations of the S-fertilized wheat, at moderate and high rates. The more detailed view on the quantitative protein composition subunits showed that admittedly the S-poor proteins like HMW-GS and ω-gliadins decreased, while S-rich proteins like LMW-GS and γ-gliadins increased. However, the sum of both gliadins and glutenins did not markedly change (**Figure 5A**,**B**). The plant's ability to maintain a certain concentration of storage proteins leads to a fine regulation that will be triggered by the S availability of the wheat plant. The storage protein formation process at kernel development should result in a well-equipped seed that may differ in the composition but not the total content of S-rich and S-poor proteins. This compensating trend is clearly obvious in both wheat cultivars differing in baking quality. Furthermore, a remarkable phenomenon supports this hypothesis; the moderate S fertilization rate leads to nearly equal concentrations of S-rich LMW-GS and S-poor HMW-GS in both wheat cultivars (**Figure 5E**,**F**), which indicated the compensational effect of S fertilization and the regulation of the development of storage proteins. The high variability of storage protein formation and the plasticity of regulation of the HMW-GS revealed a strategy for the fine-tuning effect for the adaptation mechanism of the S-dependent storage protein formation.

The effect of late S fertilization on gluten proteins was primarily detectable by comparison of the composition of glutenin subunits. Significantly higher S-rich LMW-GS concentrations for the cultivar Batis, but not for Türkis, were observed at the late S fertilization treatment (**Figure 5E**, **F**). On the other hand, the cultivar Türkis showed significantly decreased S-poor HMW-GS and ωb-gliadin concentrations at late S fertilization. Significant differences of the gliadin concentrations of S-poor ω1,2- and ω5-gliadins were restricted to the cultivar Türkis and showed higher concentrations at late S fertilization. In general, with S fertilization, S-rich gluten proteins significantly increased (*7*). These findings may have an impact on the baking quality of Batis with late S fertilization.

Proteome Analysis. In addition to the RP-HPLC analysis of gluten proteins, the use of the 2D-GE technique allows the analysis and visualization of additional nonstorage proteins with high resolution and the option of identifying single proteins changed under different S regimes. Furthermore, the physiological effect of late S fertilization might be better assessed after characterization of individually changed protein spots. By proteome analysis, ca. 380 proteins were analyzed and a set of 11−14% of total proteins differed markedly under late S fertilization in both cultivars (**Figure 6**). Most of these proteins (81% with Batis and 76% with Türkis; **Figure 6**, arrow upward) were up-regulated after late S fertilization, but some were down-regulated (18% in Batis and 24% in Türkis; **Figure 6**, arrow downward). After a mass spectrometric analysis (MALDI-TOF) and a subsequent database search, it was possible to clearly identify 81 proteins from the spots picked from the 2D gel. Unfortunately, the majority of changed proteins did not lead to validated matches in the database because of a lack of information for wheat proteins, resulting in most of the

changed proteins remaining unidentified. Therefore, in this report, the identification of changed proteins is limited to four distinct proteins: the HMW-GS 1Bx7, the HMW-GS 1By9, and one unspecified HMW-GS, as well as the starch synthase increased in both cultivars with late S fertilization (**Figure 6B** and **Table 1**). Both cultivars Batis and Türkis contain HMW-GS 1Dx5, 1Bx7, 1By9, and 1Dy10. Usually, the HMW-GS 7 is most abundant in wheat cultivars showing the HMW-GS pattern 5, 7, 9, and 10 (*39*). HMW-GS 7 is an x type HMW-GS, which has been shown to positively influence the protein quality (**Figure 6** B) (*39*). Thus, increasing the concentration of HMW-GS 7 is likely to have a positive impact on the baking performance.

Baking Quality. Cysteine residues in the protein structure are responsible for the formation of intra- and intermolecular disulfide bonds. Monomeric gliadins comprised only intramolecular bonds, while the polymeric glutenins are formed by intermolecular disulfide bonds (*40*). This effect results in a changed impact of the gliadin and glutenin fractions in terms of baking quality. The balance between viscosity (gliadins) and elasticity (glutenins) is an important factor of the baking quality (*3*). With S fertilization, S-poor proteins like HMW-GS and ω-gliadins decreased (except of HMW-GS at S deficiency), while S-rich proteins like LMW-GS and γ-gliadins increased. This resulted in a remarkable shift of the gliadin/glutenin ratio and thus improved the baking quality of both cultivars (**Figure 7**). Late S fertilization led to a shift of the synthesis of gluten proteins, which resulted in a shift of gliadin and glutenin attributes. The significant increase of S-rich LMW-GS concentrations of the late S fertilization is responsible for the effect of improved baking quality (**Figures 5E** and **7**). Besides the differences in gluten proteins caused by late S fertilization, a general difference between the cultivars was detected in the vice versa relation of α-gliadins and γ-gliadins with higher concentrations of γ-gliadins in Batis and higher concentrations of α-gliadins in Türkis (**Figure 5E**,**F**). In terms of crude protein concentration, sedimentation value, and dough elasticity, the cultivar Türkis was classified in the same baking quality class as Batis according to the German Federal Office of Plant Varieties (*16*). However, with the parameter bread volume included, the cultivar Türkis is classified in a higher rank. Nevertheless, no significant difference between the cultivars concerning baking quality was detected (**Figure 7B**).

In conclusion, adequate S fertilization together with high N supply has an impact on S concentration, GSH concentration, certain S-rich gluten proteins, and the protein profile plus the baking quality of wheat grain. Farm and food quality profits may be influenced by improved S availability and timely fertilization during grain development. Late S fertilization significantly changed certain proteins, such as HMW-GS, and benefited baking quality.

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Supporting Information Available: RP-HPLC chromatograms of the cultivar Türkis. This material is available free of charge via the Internet at http://pubs.acs.org.

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