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

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Highlights:

- Increasing protein concentration does not necessarily mean better baking quality
- Baking performance depends on composition of storage proteins
- Late foliar N application improves baking quality when N supply is low

23   • For baking quality evaluation, protein composition and bread volume are

24   required

25 Declaration of interest: none

26   Abstract

27   Wheat is the second biggest staple crop worldwide and is mainly consumed in the

28   form of baked goods, requiring a specific flour quality. Grain protein concentration

29   (GPC) is an underpinning parameter for baking quality and therefore strongly

30   influences the value of wheat. It is a common strategy to increase GPC by combining

31   high protein varieties with the application of a late dose of nitrogen. However, the

32   late use of N fertilisers can cause environmental problems, such as nitrate leaching

33   and gaseous losses. Furthermore, recent studies show, that there is only a weak

34   relationship between GPC and bread volume. The aim of this study was to re-

35   evaluate the merits of a late N application by investigating the effects of a late foliar

36   N application, not only on GPC, but also on the gluten protein composition and on

37   bread volume.

38   In this study, an increasing GPC did not necessarily lead to improved baking quality.

39   Baking performance rather depended on the grain protein composition. Only at a low

40   N fertiliser level (100 kg N ha^{-1}), the foliar N application decreased the HMW-

41   GS/LMW-GS ratio and increased the gliadin/HMW-GS ratio, which led to an

42   improved bread volume. These results imply that a late foliar N application can be

43   used to effectively improve baking quality when the total N uptake was low due to

44   low fertilisation or unfavourable weather conditions. The results also show that

45   quality cannot be evaluated by measuring GPC alone but also needs information of

46   storage protein composition as well as bread volume.

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

48   **Abbreviations:** GPC, Grain protein concentration; HMW-GS, high molecular weight

49   glutenin subunits; LMW-GS, low molecular weight glutenin subunits; TGW, 1000

50   grain weight; HFN, Hagberg falling number; NABIM, National Association of British

51   and Irish Millers

1. Introduction

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

The late use of N fertilisers can cause environmental problems, such as nitrate leaching into the groundwater and gaseous losses in the form of nitrous oxide, which contribute to global warming (Senbayram et al., 2009). For farmers, these problems

77 represent a monetary risk, particular in the case of unfavourable weather conditions

78 which may limit the uptake of the supplied nitrogen and hence, the required GPC

79 might not be reached. In this case, a large amount of N remains in the soil and

80 increases the environmental risks mentioned above. In this study, the method of

81 foliar N application was chosen as an alternative as it holds advantages over the soil

82 application of N fertilisers. Effects of N fertiliser management on GPC, grain protein

83 composition and baking quality have been investigated previously (Pechanek et al.,

84 1997; Wieser and Seilmeier, 1998; Wan et al., 2014; Schulz et al., 2015; Xue et al.,

85 2016a; Xue et al., 2016b), but the observed changes in protein composition due to

86 different N fertilisation managements and their influence on baking quality are

87 inconsistent. Furthermore, the influence of a foliar N application was considered in

88 none of these studies. Woolfolk et al. (2002), Bly and Woodard (2003) and Tea et al.

89 (2004) studied the influence of a foliar N application on various quality parameters,

90 but not on bread volume. For example Wieser and Kieffer (2001) found a strong

91 influence of the gliadin/glutenin ratio on rheological properties and bread volume, but

92 this could not be confirmed by Pechanek et al. (1997). Another example is the

93 influence of N fertilisation on the quantity of HMW-GS, which was reported to be

94 positive (Wieser and Seilmeier, 1998) and negative (Pechanek et al., 1997). As the

95 relationship of protein composition and baking quality remains unclear, further

96 investigations are needed.

97 In this context, the aim of this study was to re-evaluate the merits of a late N

98 application by investigating not only the effects of a late foliar N application on GPC,

99 but also on the composition of grain proteins and on the bread volume, in three

100 wheat cultivars, under two N fertiliser regimes.

101

2. Material and Methods

2.1.   Plant cultivation

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

127   2.2.   Yield, N, GPC and N-harvest index

128   Plants were harvested from a sample area (0.5 m²) in each plot at anthesis and at

129   maturity. Mature plants were threshed and grain and straw fresh and dry weight were

130   recorded as well as thousand-grain-weight (TGW) and Hagberg Falling number (FN).

131   Yields are expressed as 85 % dry matter (DM). The weight of flour used for Hagberg

132   Falling Number measurement was adjusted according to the moisture content of the

133   flour. Grain and straw samples were ground with an Ultra Centrifugal Mill (ZM 200,

134   Retsch) and a Hammer Mill (Christy & Norris 8" Lab Mill, Christy Turner Ltd) for

135   further analysis. The nitrogen content of grain and straw samples was measured by

136   the Dumas method using a LECO CN628 Combustion Analyser (LECO Corporation,

137   St Joseph, Michigan, USA) and is expressed in percent of dry matter. Grain protein

138   concentration was calculated by multiplying the N concentration by the factor 5.7.

139   Elemental analyses of grain and straw samples were carried out using ICP-MS

140   (inductively coupled mass spectrometry). Nitrogen harvest index (N-HI) was

141   calculated according to the following equation:

142
$$NHI\ [\%] = \frac{Grain\ N\ content}{Shoot\ N\ content} * 100$$

143   2.3.   Micro baking test

144   The moisture content of flour samples was calculated from the weights of a given

145   amount of flour before and after drying for 2 hours at 110 °C. Afterwards, 10 g of

146   wholemeal flour (14.0 % moisture) were mixed with 0.2 g NaCl and then used to

147   determine optimal water uptake and optimal dough kneading time by means of a

148   farinograph (Farinograph-E, Brabender GmbH & Co. KG, Duisburg, Germany). An

149   optimal dough development was defined at a dough consistency of 550 Brabender

150   Units (530-570 BU) (Kieffer et al., 1998). For the microscale baking test, 10 g of flour

8

151 (14.0 % moisture) were combined with 0.7 g fresh yeast and 0.1 g shortening. 1 mL

152 NaCl/sucrose-solution (1 % NaCl, 2 % sucrose), 0.3 mL 0.004 M L(+)-ascorbic acid

153 solution and the determined amount of water were added and the dough was

154 kneaded for the optimal time required to reach 550 BU. After kneading, the dough

155 was left to rest at 30 °C and 90 % relative humidity for 20 minutes. The dough was

156 reshaped and rounded before secondary proofing for 40 minutes. Finally, the dough

157 was baked for 10 minutes at 180 °C increasing to 250 °C on an automated proofing

158 baking line. The bread loaves were weighed after cooling and bread volume was

159 measured by surface scanning, using a Volscan Profiler 300 (Stable Micro Systems

160 Ltd, Godalming, UK).

161   2.4.   Protein extraction and SDS-PAGE

162 Grain samples taken at five time-points after anthesis (10, 14, 21, 28, 35 DPA) were

163 ground using a freezer mill (Freezer Mill 6870, Spex Sample Prep). Aliquots of each

164 sample were freeze-dried for at least 36 hours. Total gluten protein fraction was

165 extracted from 10 mg flour using 50% propan-1-ol + 2% dithiothreitol (DTT) at 50 °C,

166 the extraction being repeated and the supernatants combined. After freeze-drying,

167 the protein was dissolved in lysis buffer (8 M urea, 2 M thiourea, 4 % CHAPS, 30

168 mM DTT, 20mM Tris base). The 2-D Quant Kit (GE Healthcare) was used to quantify

169 the extracted protein. The sample amount containing 10 μg protein was combined

170 with loading buffer (50 mM Tris-HCl (pH 6.8), 2 % SDS, 10 % glycerol, 0.1 %

171 bromophenol blue, 200 mM DTT) and loaded on 7 cm precast gels (4-15% Mini-

172 PROTEAN TGX Precast Protein Gels, Biorad, Munich, Germany) for 1D SDS-PAGE

173 (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Precision Plus

174 Protein[TM] Standard (Biorad) was applied to each gel as protein ladder. Running

175 conditions were 40 V for 30 minutes, then 80 V for 120 minutes. After completion of

176 the run, gels were fixed in a fixing solution (40 % ethanol, 50 % H_2O and 10 % acetic

177 acid) for one hour. Afterwards, gels were stained with staining solution (1 Coomassie

178 tablet (GE Healthcare, Freiburg, Germany) in 1.6 L 10 % acetic acid) for one hour

179 and later, destained in 10 % acetic acid overnight. Gels were then scanned using an

180 Epson Perfection V700 scanner. Gel image analyses were carried out using the

181 GelAnalyzer 2010a software (gelanalyzer.com). The protein bands were classified

182 into three groups according to He et al. (2013) and Wan et al. (2014). The first group

183 corresponded to the HMW-GS, the second group to the ω-gliadins and the third

184 group combined a mixture of LMW-GS, and α-/γ-gliadins.

185   2.5.   Protein extraction and SE-HPLC

186 For SE-HPLC (size-exclusion high performance liquid chromatography) analysis,

187 16.5 mg wholegrain flour were suspended in 1.5 mL of 5% SDS in sodium

188 dihydrogen phosphate (pH 6.9) and then stirred for 5 minutes. Samples were then

189 sonicated for 40 seconds, followed by centrifugation (30 min at 10000 g) to obtain

190 the proteins in the supernatant. Separation was achieved in 30 min by loading 20 µL

191 of sample into an eluent (50% (v/v) acetonitrile and water, containing 1 mL L^{-1}

192 trifluoroacetic acid (TFA)) at a flow rate of 0.2 mL min^{-1} using a Shimadzu

193 Prominence HPLC and a Phenomenex BioSep™ SEC s4000 column. Proteins were

194 detected by UV absorbance at 210 nm. The SE-HPLC chromatograms were divided

195 into five sections of decreasing molecular size according to (He et al., 2013): large

196 glutenin polymers (F1), small glutenin polymers (F2), ω-gliadins (F3), α-/γ-gliadins

197 (F4) and non-gluten proteins (F5).

198   2.6.   Statistical analysis

199 The GenStat (17th edition, VSN International Ltd., Hemel Hempstead, UK) software

200 was used for statistical analyses. Data were analysed by ANOVA, with the design

201 structure of the experiment (randomised plots) being taken into account.

202 Comparisons of relevant means were made using the least-significant difference at

203 the 5 % level (p=0.05). For the statistical analysis of the gel electrophoresis data, in

204 addition to the field structure, the structure of the laboratory analysis (technical

205 replicates) was included. For statistical analysis, the protein composition data from

206 SDS-PAGE were converted to square root scale to assure variance homogeneity.

207 Regression analysis was used to evaluate the relationships between grain protein

208 concentration, gluten protein or the amount of gliadins and bread volume.

3. Results

3.1. Grain yield and GPC

211 Three varieties were grown in a randomised field experiment at Rothamsted, UK at

212 two fertilisation levels of nitrogen (100 kg N ha^{-1} and 200 kg N ha^{-1}). An additional

213 dose of foliar N was applied at anthesis to one half of each plot. The three tested

214 wheat varieties belong to different quality groups. Skyfall is a high yielding bread

215 wheat with consistent quality in NABIM (National Association of British and Irish

216 Millers) group 1. Soissons is an early maturing variety from NABIM group 2.

217 Conqueror is a hard-milling feed wheat and belongs to NABIM group 4. Grain yield

218 was enhanced by a higher N fertiliser level in both years except for Soissons in 2017

219 (Figure 1 A). On average, the higher N fertiliser level resulted in an additional grain

220 yield of 2.9 and 1.2 t ha^{-1} in 2016 and 2017, respectively. An increased grain yield

221 due to foliar N application at anthesis was only observed for Skyfall at N100 and for

222 Soissons at N200. In 2016 at the 100 kg N ha^{-1} level, the grain yield of the feed

223 wheat cultivar Conqueror was significantly (p<0.05) higher than that of the bread

11

224 making cultivar Soissons, but there were no cultivar differences observed at the 200

225 kg N ha^{-1} level. In 2017, Soissons had a lower grain yield than Conqueror and Skyfall

226 at both N fertilisation levels. GPC (calculated as grain N concentration x 5.7) was

227 increased by a higher N fertiliser level as well as foliar N application at anthesis

228 (Figure 1 B). Without foliar N application at anthesis, the average grain protein

229 concentration was 8.9 %, which was raised to 10.3 % by foliar N application. There

230 were significant ($p < 0.05$) differences in GPC between cultivars. The lowest grain

231 protein concentration was observed for Conqueror with 8.7 %, which was expected

232 as Conqueror is a feed wheat. Contrary to the NABIM classification, GPC of

233 Soissons (10.6 %) exceeded that of Skyfall (9.6 %). However, considering the

234 average GPC, both varieties did not reach the standard specification for bread-

235 making of at least 12 %. This minimum GPC for bread-making wheat was only

236 achieved by Soissons in 2017 at the high N level (N200) combined with the foliar N

237 application. GPCs for all varieties and N fertiliser levels were lower in 2016 than in

238 2017.

239 GPCs achieved at N200 + foliar N ranged from 9.8 % (Conqueror) to 11.9 %

240 (Soissons) in year 2016 and from 11.2 % (Conqueror) to 13.5 % (Soissons) in year

241 2017. The minimum protein concentration of 12 %, required for bread-making in the

242 U.K. (Godfrey et al., 2010; He et al., 2013) was only reached by the two bread-

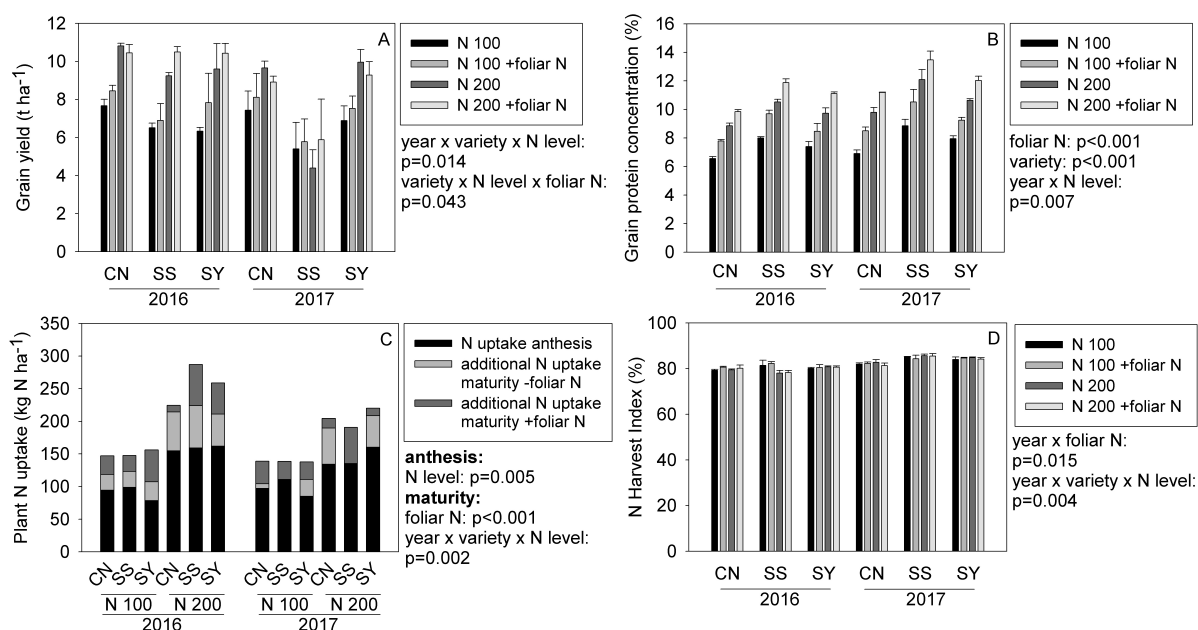243 making wheat varieties tested in one of the two years.

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

3.2.   Plant N uptake and nitrogen harvest index

Plant N uptake was calculated from N concentration and biomass data at anthesis and maturity; the results are presented in Figure 1 C. At anthesis plant N uptake was equal for all three varieties with higher N uptake at the higher N fertiliser level. For N100, plants absorbed 94 kg N ha^{-1} on average until anthesis and 151 kg N ha^{-1} on average for N200. When no foliar N was applied, the average additional N uptake post anthesis was 16.7 and 42.5 kg ha^{-1} for N100 and N200, respectively. When foliar N was applied, the additional N uptake post anthesis increased to 48.6 kg ha^{-1} at N100 and to 76.3 kg ha^{-1} at N200. An additional foliar N application at anthesis increased N uptake at maturity by 33 kg ha^{-1}. This is consistent with the foliar-applied
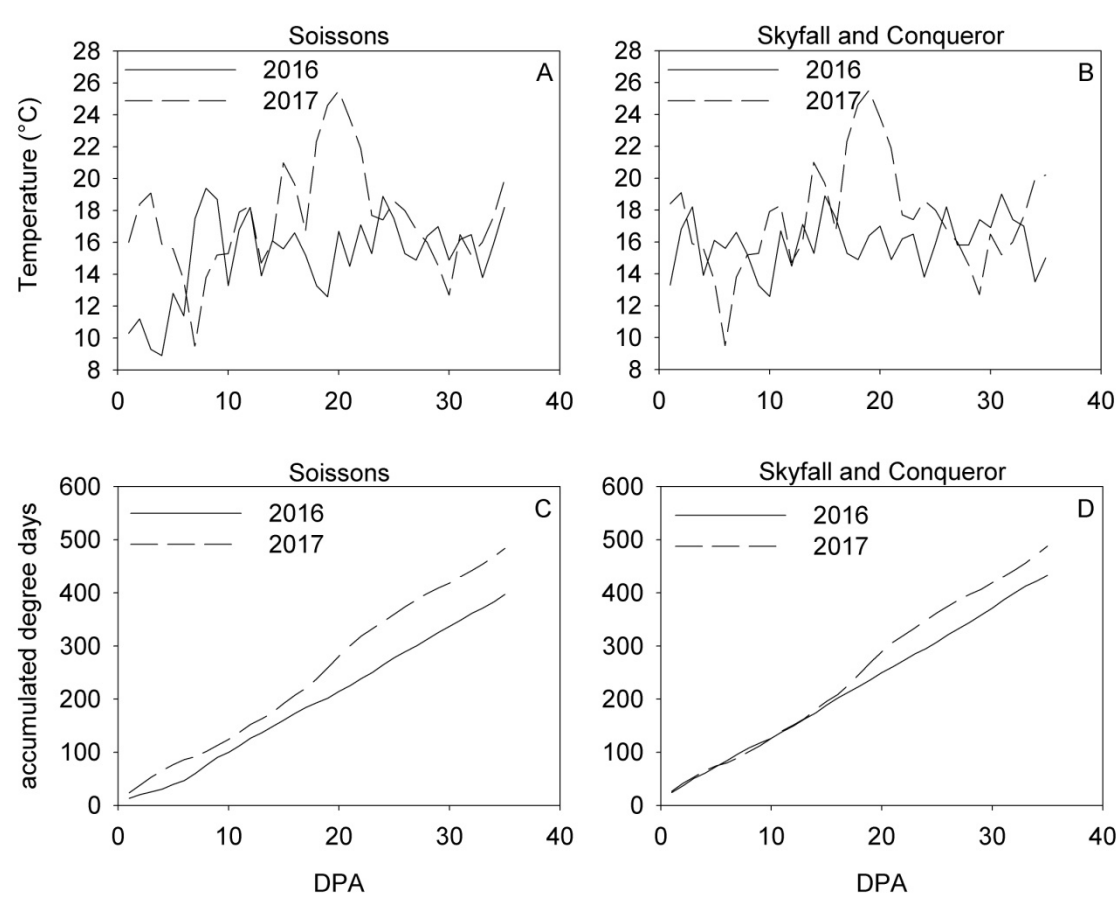
13

260   40 kg ha^{-1} N at anthesis. The higher N fertiliser regimen resulted in significantly

261   ($p < 0.05$) higher plant N uptake at maturity in all three cultivars.

262   The N-HI (nitrogen harvest index) was enhanced by a foliar N application at anthesis

263   only in 2016. With an average of 80.1 %, the N-HI in 2016 was lower than in 2017

264   (83.9 %) as is shown in Figure 1 D. Statistical analysis showed a three-way

265   interaction of the factors year, cultivar and N level on the N-HI. Part of this interaction

266   is a decrease of the N-HI due to a higher N fertilisation level, but this effect only

267   appeared in the cultivar Soissons and only in year 2016. In that year, Soissons had a

268   higher N-HI than Skyfall and Conqueror at N100, but at N200 the N-HI for Soissons

269   was the lowest. In 2017, the lowest N-HI was observed for Conqueror (82.1 %).

270   There was no difference between Skyfall and Soissons at N100, but at N200 the N-

271   HI of Soissons (85.6 %) was higher than that of Skyfall (84.5 %).

272   3.3.   Weather conditions during grain development

273   Weather conditions after anthesis in 2017 differed from those in 2016. Weather data

274   for the variety Soissons and the varieties Skyfall and Conqueror are presented

275   separately (Figure 2 and Figure 3) because in year 2016 Soissons' development was

276   approximately one week earlier than the development of Skyfall and Conqueror. In a

277   period from 15 to 23 DPA for Soissons and from 17 to 22 DPA for Skyfall and

278   Conqueror, daily average temperatures were higher in 2017 than in 2016 (Figure 2

279   A). This led to higher accumulated degree days in 2017 from 17 DPA onwards for

280   Skyfall and Conqueror, for Soissons the accumulated degree days in 2017 lay above

281   those in 2016 for the whole period that was monitored (Figure 2 B). The accumulated

282   rainfall and duration of sunshine during grain development are displayed in Figure 3.

283   Both parameters show considerable differences between the two years of the

284   experiment. In 2017, the total rainfall with 39 mm in a period from anthesis until 35

285   DPA was less than in 2016 (97 mm for Soissons and 118 mm for Skyfall and

286   Conqueror). The duration of sunshine in the same period was 273 h for Soissons

287   and 287 h for Skyfall and Conqueror in 2017, while in 2016 it was only 171 h

288   (Soissons) and 220 h (Skyfall and Conqueror). For Soissons in 2017 there was 58

289   mm less rainfall and 102 more hours of sunshine during grain development

290   compared to 2016. For Skyfall and Conqueror, there was 79 mm less rainfall and an

291   additional 67 h of sunshine in 2017 in comparison with 2016.

292

293   Figure 2: Daily average temperature (A and B) and accumulated degree days (C and

294   D) during grain development (1-35 days post anthesis (DPA)) of the cultivars

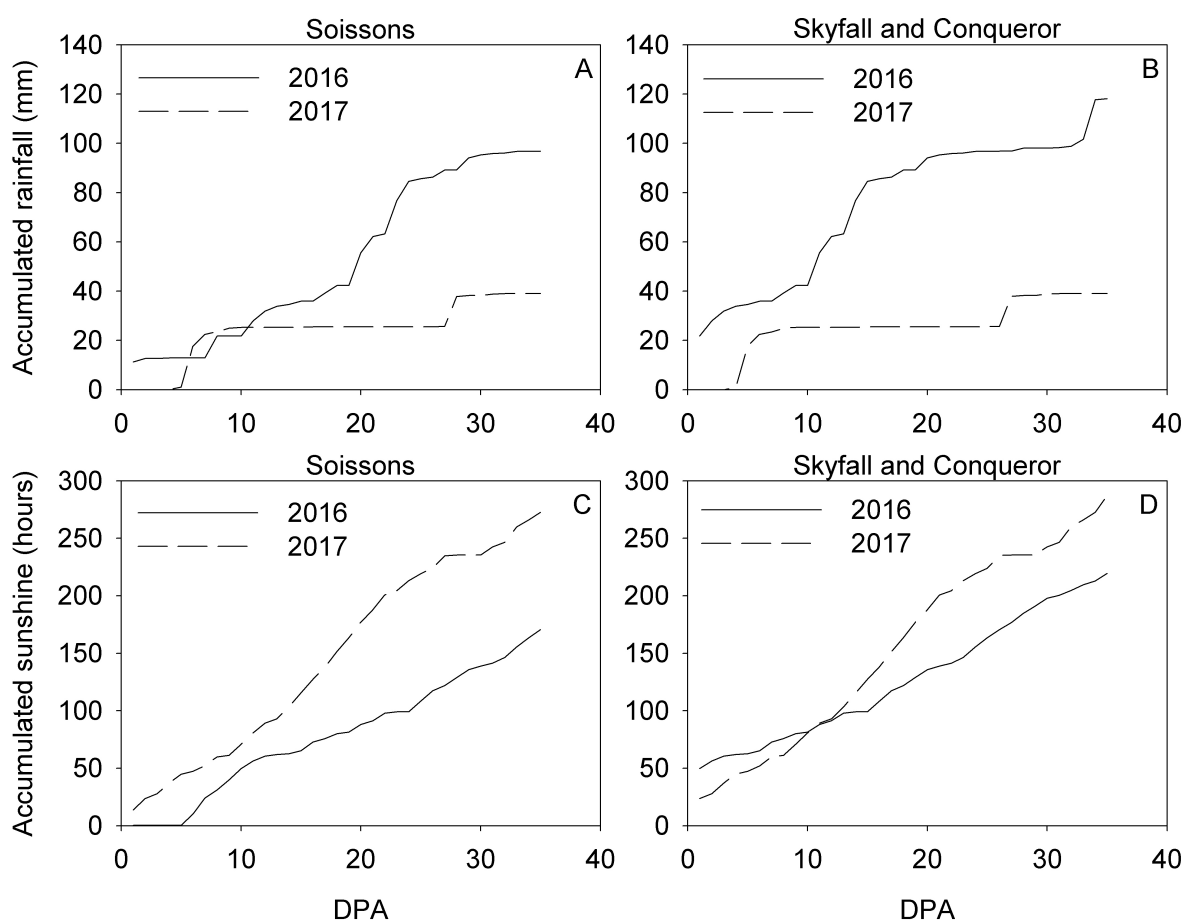295   Conqueror, Soissons and Skyfall in the years 2016 and 2017.

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

3.4. Thousand-grain-weight and Hagberg falling number

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

16

308 levels, Soissons had the smallest grains with an average TGW of 37.72 g. TGW for

309 Conqueror and Skyfall did not differ at N100, but at N200 Skyfall with a TGW of

310 46.37 g, had bigger grains than Conqueror (43.47 g). As TGW varied between

311 varieties but yield was similar, it may be concluded that those varieties with smaller

312 grains (lower TGW), such as Soissons, produced more kernels to compensate for

313 the smaller size of grains.

314 Hagberg falling number (HFN) is a measure of the starch quality of flour produced

315 from wheat grain. A falling number of less than 220 s is recognised as low, whereas

316 a falling number greater than 300 s is high. The HFN was improved by 23.5 % due to

317 the foliar N application at anthesis when N supply was low (N100) but at high N

318 supply (N200) there was no effect of foliar N on HFN observed (Figure 4 B). A higher

319 N fertiliser level only improved HFN when no foliar N was applied at anthesis. There

320 was a strong gradient within the three tested varieties, with flour from Skyfall having

321 the highest HFN (350.7 s), followed by flours from Soissons (318.1 s) and Conqueror
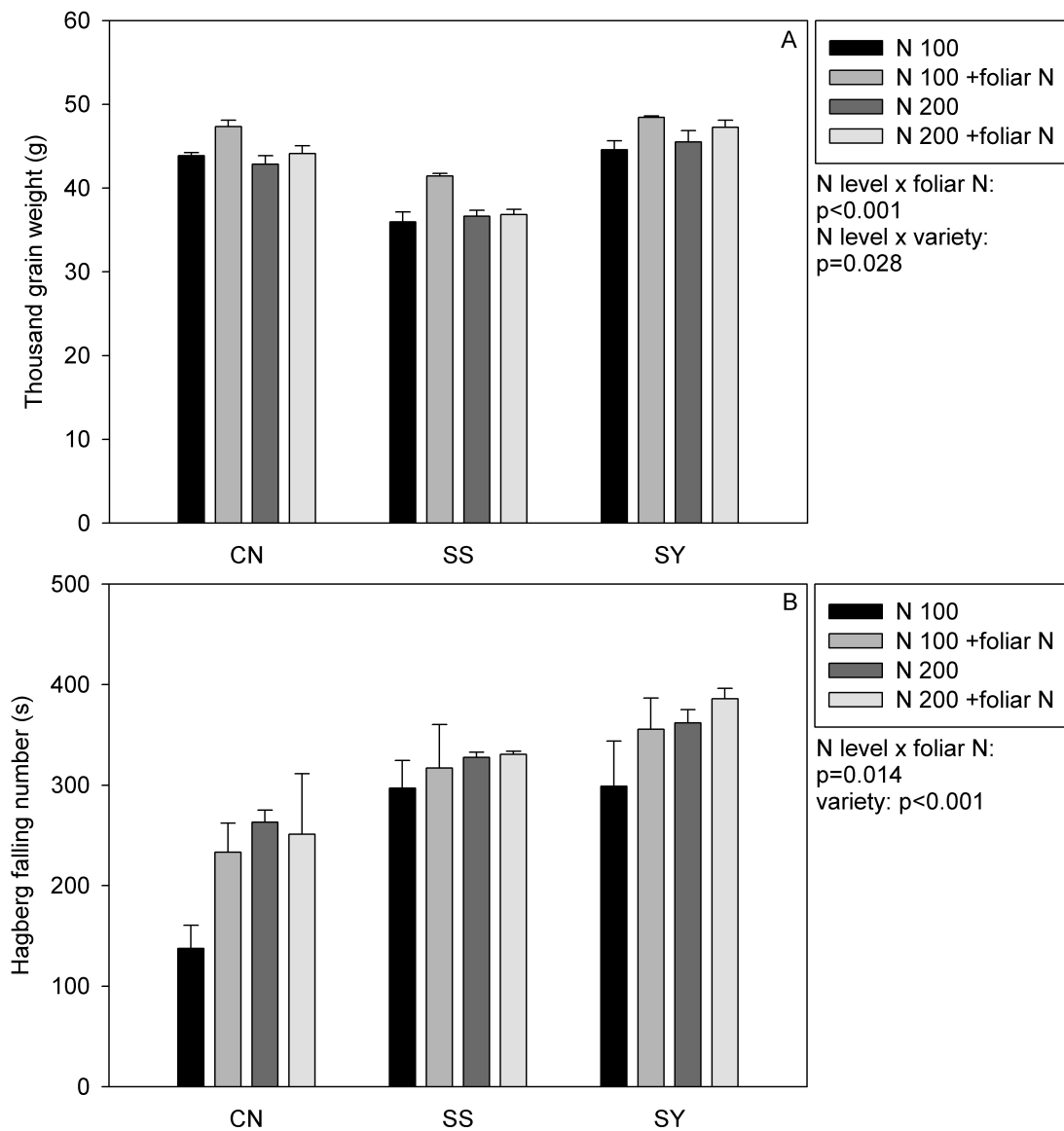
322 (221.4 s).

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

3.5. Bread volume

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

18

332   Conqueror (24.28 and 23.36 mL, respectively). A foliar N application at anthesis

333   improved bread volume only at the low N fertilisation level (N100). In general, bread

334   volumes were higher at the high N fertilisation level (N200). The average bread

335   volume at N100 with an additional dose of N applied via the leaves at anthesis

336   (25.78 mL) was comparable with the average bread volume at N200 without a foliar
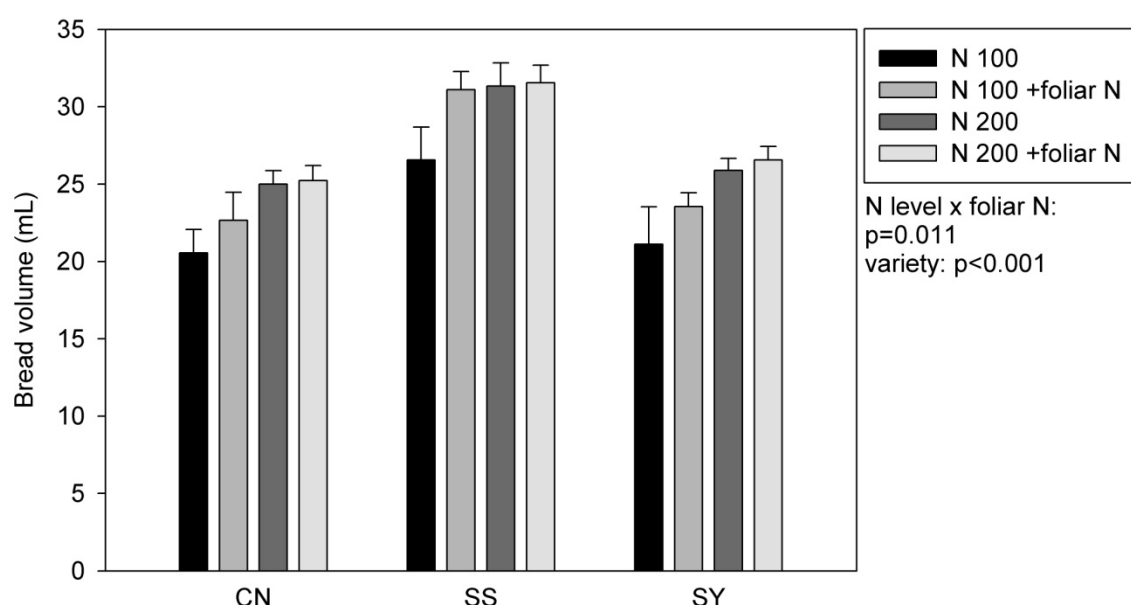
337   N application at anthesis (27.41 mL).

338

339   Figure 5: Bread volume in the cultivars Conqueror, Soissons and Skyfall as affected

340   by the two N fertilisation levels 100 kg N ha^{-1} (N100) and 200 kg N ha^{-1} (N200), with

341   and without foliar N application at anthesis. Error bars represent standard deviations

342   of replicates.

343   3.6.   Protein composition, measured by SDS-PAGE

344   Extracted grain proteins were separated by size on the basis of their mobility using

345   SDS-PAGE to analyse the gluten subunits. The protein bands were classified into

346   three groups. The first group corresponded to the HMW-GS, the second group to the

347   ω-gliadins and the third group combined a mixture of LMW-GS, and α-/β-/γ-gliadins.

348   The SDS-PAGE results are shown in Figure 6 A.

349   Considering the general increase in GPC with higher N fertiliser level and foliar N

350   application at anthesis, the alterations in the composition of the storage protein

351   fractions given as percentage from total extracted protein is of particular interest. The

352   LMW-GS and α-/β-/γ-gliadins were the major group of gluten proteins with an

353   average of 90.7 %. The HMW-GS made up for 6.5 % whereas the ω-gliadins

354   accounted for 2.8 %. The percentage of ω-gliadins was not changed by foliar N

355   application at anthesis. The percentage of HMW-GS was increased by foliar N

356   application while the percentage of LMW-GS and α-/β-/γ-gliadins was decreased.

357   The gluten protein composition was also influenced by variety. Soissons showed

358   higher percentages of HMW-GS and ω-gliadins than Skyfall and Conqueror. The

359   percentage of LMW-GS and α-/β-/γ-gliadins was lowest in Soissons (82.6 %) and
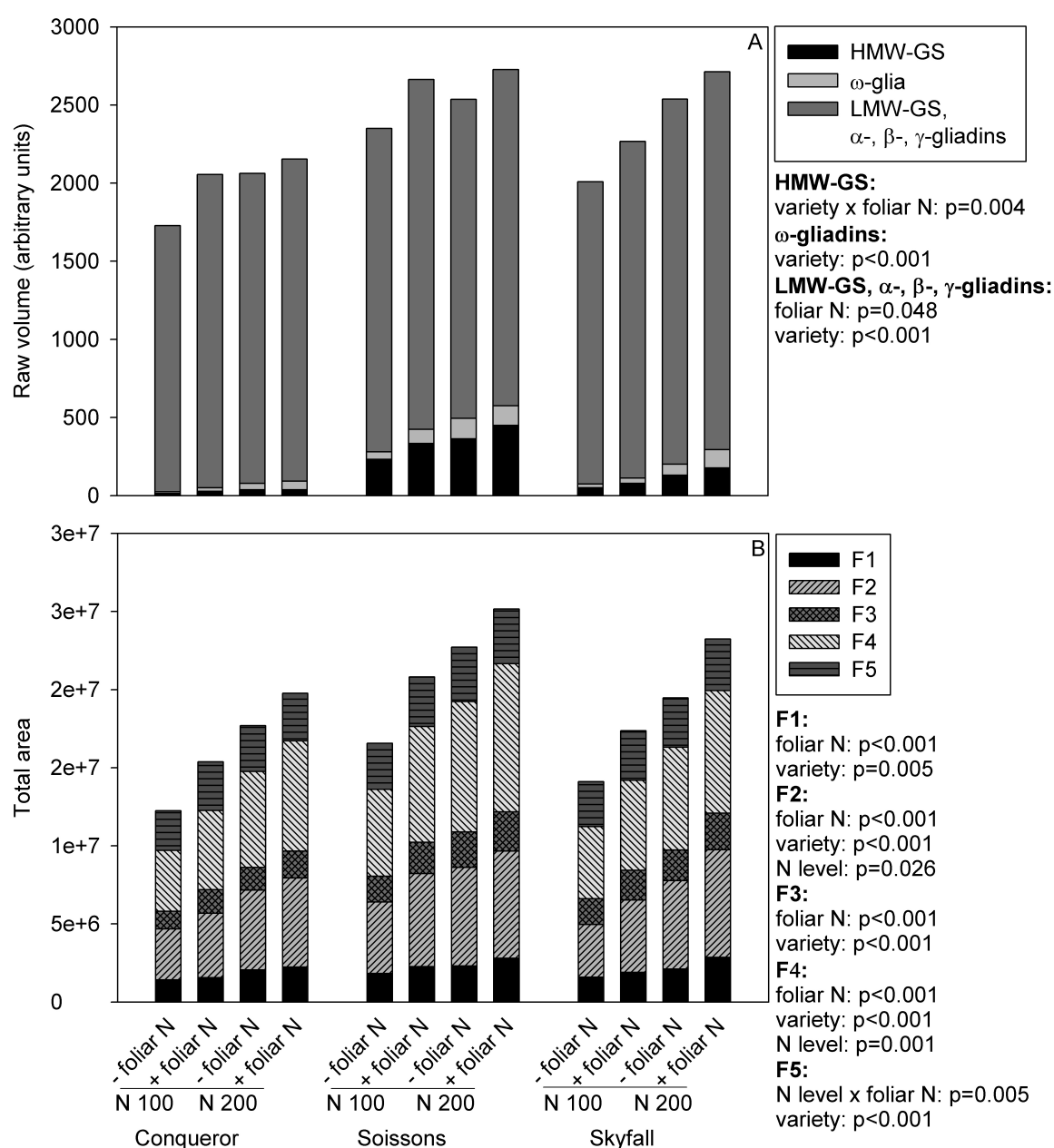
360   highest in Conqueror (96.6 %).

Figure 6: Proportions of protein fractions at maturity, measured by SDS-PAGE (A) and composition of protein polymers analysed by SE-HPLC (B) (displayed as area underneath the chromatogram), as affected by the two N fertilisation levels 100 kg N ha^{-1} (N100) and 200 kg N ha^{-1} (N200), with and without foliar N application at anthesis in the cultivars Conqueror, Soissons and Skyfall. **F1**: large glutenin polymers (containing HMW-GS), **F2**: small glutenin polymers (containing LMW-GS),

21

368   **F3**: fraction enriched in ω-gliadins, **F4**: fraction enriched in α-/γ-gliadins and **F5**:

369   containing non-gluten proteins.

370   3.7.   Protein polymer composition, analysed by SE-HPLC

371   Storage proteins were analysed by size using SE-HPLC technique to analyse

372   polymers. The results from this analysis are presented in Figure 6 B. The SE-HPLC

373   chromatograms were divided into five sections, representing different storage protein

374   fractions: large glutenin polymers (high molecular weight glutenin subunits (HMW-

375   GS)) (F1), small glutenin polymers (low molecular weight glutenin subunits (LMW-

376   GS)) (F2), ω-gliadins (F3), α-/γ-gliadins (F4) and non-gluten proteins (F5).

377   (F1+F2+F3+F4+F5) represents the total protein amount, whereas (F1+F2+F3+F4)

378   represents the amount of gluten protein.

379   While the total amounts of HMW-GS, LMW-GS, ω-gliadins and α-/γ-gliadins were

380   increased by a foliar N application at anthesis at both, low and high N fertilisation

381   level, the amount of the non-gluten proteins was increased solely at the low N

382   fertiliser regimen (Figure 6 B). Only the LMW-GS and α-/γ-gliadins were enhanced

383   by a higher N fertilisation level. For all storage protein groups, there was a gradient

384   between the three tested cultivars, with Conqueror showing the least amounts of

385   each protein group, followed by Skyfall and then Soissons with the highest amounts.

386   The HMW-GS are the only exception from this pattern, with Skyfall and Soissons

387   showing similar amounts, both higher than Conqueror.

388   Ratios between certain storage protein fractions, such as HMW-GS/LMW-GS ratio,

389   gliadin/glutenin ratio and gliadin/HMW-GS ratio ((F3+F4)/F1), are said to indicate for

390   the baking quality (Godfrey et al., 2010; Millar, 2003). The ratio of HMW-GS to LMW-

391   GS is given by F1/F2, the gliadin/glutenin ratio is calculated by (F3+F4)/(F1+F2) and

392   the ratio of gliadin to HMW-GS is represented by (F3+F4)/F1. Both, HMW-GS/LMW-

393 GS ratio and gliadin/HMW-GS ratio were changed due to the foliar N application at

394 anthesis, but only at the low N fertilisation level (Figure 7). The HMW-GS/LMW-GS

395 ratio was decreased, whereas the gliadin/HMW-GS ratio was increased by the foliar

396 N application. At the low N fertiliser level (N100), the amount of gliadins was

397 increased by 33 % as a result of foliar N application at anthesis, whereas the amount

398 of HMW-GS was increased by 19 %. As the increase in gliadins was stronger than

399 that of HMW-GS, the ratio of gliadins to HMW-GS was enhanced by foliar N

400 application at anthesis under a low N fertiliser regimen (Figure 7 B). The
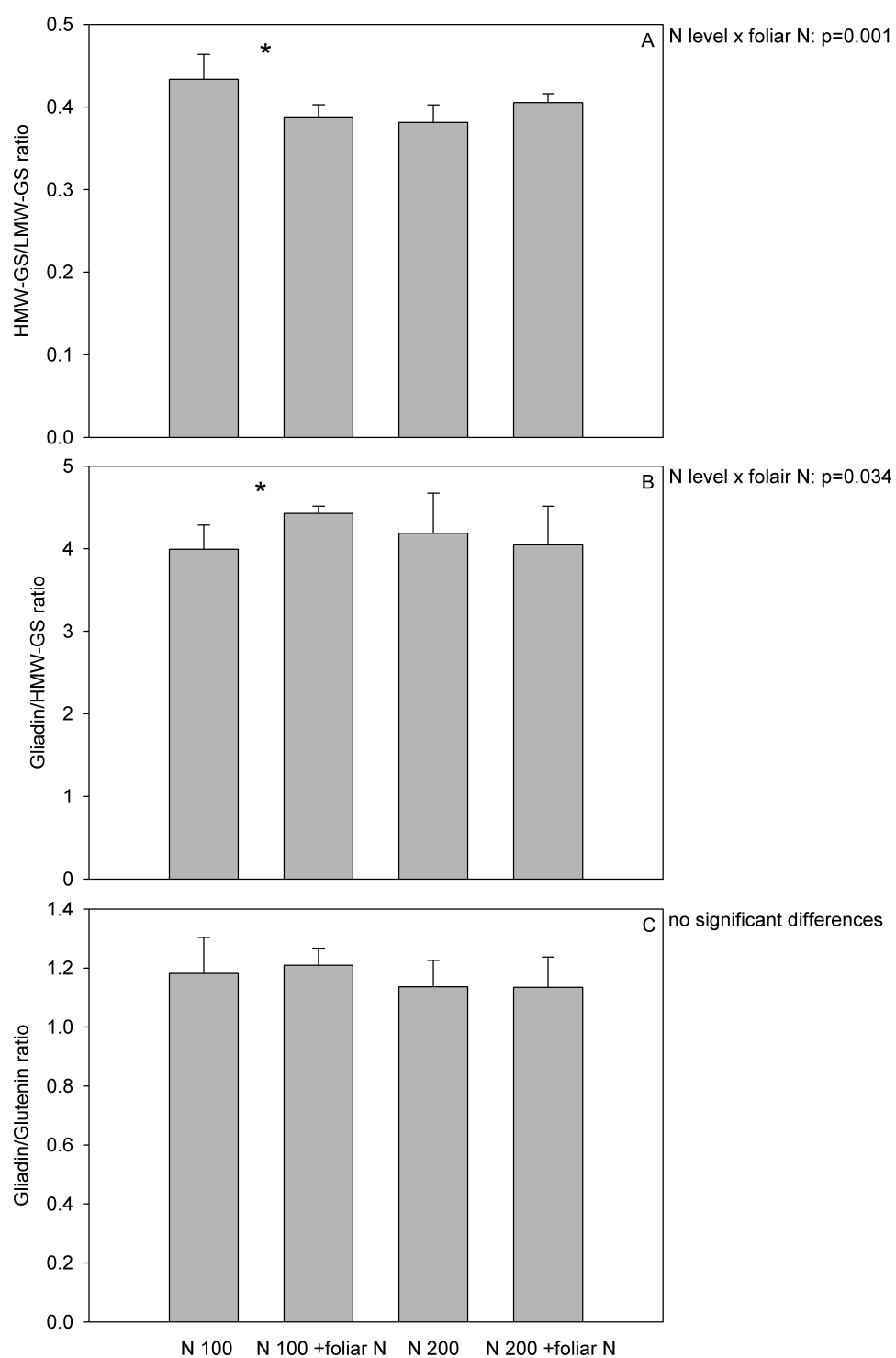
401 gliadin/glutenin ratio remained unaffected.

402

Figure 7: HMW-GS/LMW-GS ratio (A), gliadin/HMW-GS ratio (B) and gliadin/glutenin
ratio (C) at the two N fertilisation levels 100 kg N ha^{-1} (N100) and 200 kg N ha^{-1}
(N200), with and without foliar N application at anthesis. Error bars represent

406   standard deviations of replicates. Asterisks mark significant effects of a foliar N

407   application (p<0.05)

408   3.8.   Relationships between some protein-related parameters and

409   bread volume

410   Regression analyses were carried out to evaluate the relationships of all gluten

411   protein fractions and subunits measured by SE-HPLC, results obtained from

412   farinograph measurements, and in addition GPC, with bread volume. The

413   correlations of the most promising three parameters (GPC, total amount of gliadins

414   and total amount of gluten protein) are presented in Figure 8. The best correlation

415   was found between bread volume and the amount of gliadins in the flour with a

416   correlation coefficient of 0.718. When regression analyses were performed for the

417   varieties separately, the correlations were weakest for Soissons and strongest for

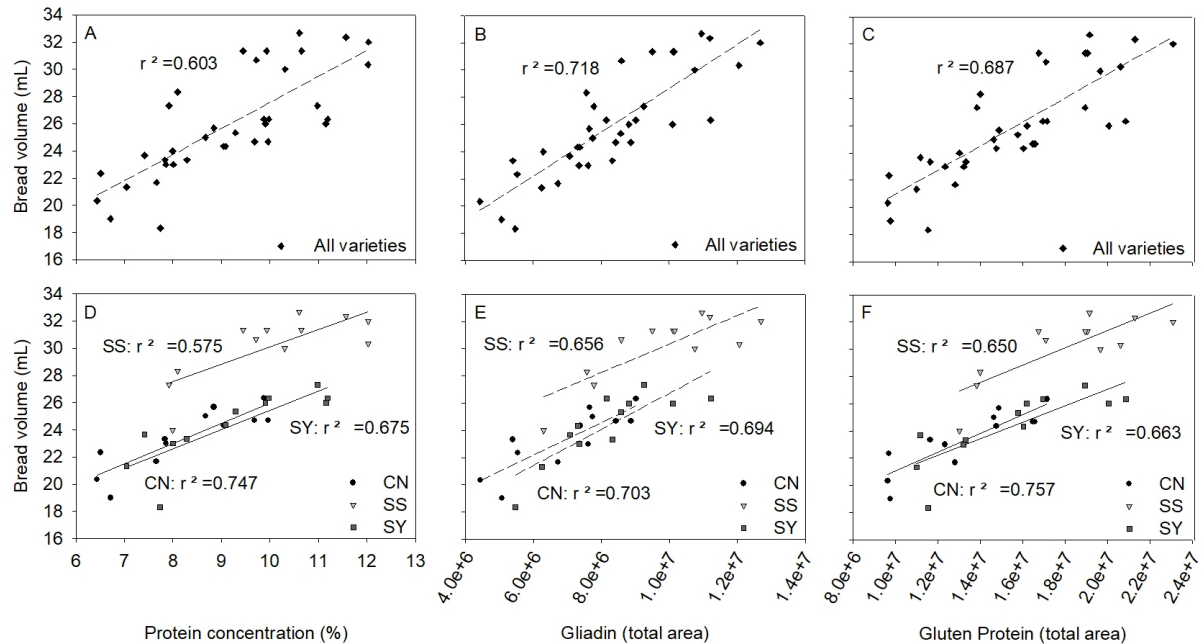418   Conqueror.

419

420   Figure 8: Correlations between bread volume and protein concentration (A and D),

421   bread volume and the amount of gliadins measured by SE-HPLC (B and E), and

422 bread volume and the amount of gluten protein measured by SE-HPLC (C and F) for

423 all three varieties (A, B and C) and for each of the three varieties (Conqueror (CN),

424 Soissons (SS) and Skyfall (SY)) (D, E and F).

425 4. Discussion

426 4.1. Plant N uptake effects on grain yield and GPC

427 As nitrogen is an essential component of grain proteins, plant N uptake is crucial for

428 grain yield production as well as storage protein accumulation during grain

429 development. When more N (N100 vs. N200) was supplied, plants assimilated more

430 N until anthesis. This plant N uptake until anthesis results in both a higher grain yield

431 and a greater GPC. The increased plant N uptake at maturity due to a foliar N

432 application at anthesis influenced grain yield only for Skyfall at N100 and for

433 Soissons at N200, but led to an increased GPC in all three tested varieties at both N

434 fertiliser levels (Figure 1). Therefore, it can be assumed that the additional N, taken

435 up after foliar N application at anthesis, was used primarily for grain protein

436 synthesis. This finding goes along with results from Kichey et al. (2007) and

437 Taulemesse et al. (2016), showing that major proportions of N absorbed during the

438 post-anthesis period are translocated to the grain. Also, Martre et al. (2006) showed

439 that especially under low N supply, the N accumulation in the wheat grain is sink-

440 regulated. Plant N uptake is an important measure, but under high N fertiliser

441 regimes, grain N concentration usually reaches a maximum and then remains stable,

442 whereas the N concentration in the straw fraction continues to rise (Barneix, 2007;

443 Kong et al., 2016; Pask et al., 2012; Triboi and Triboi-Blondel, 2002). In this case,

444 the N harvest index would be reduced. In this experiment, the higher N level only

445 decreased N-HI in Soissons in one of the two years. For the cultivars Skyfall and

446 Conqueror there was no such effect observed. These findings demonstrate that even

26

447 a dose of 200 kg N ha^{-1} can still be used effectively by wheat plants by translocating

448 the absorbed N to the grains. The changes in GPC under different N fertiliser

449 treatments also support this statement because GPC was enhanced by both a

450 higher N fertiliser level and a foliar N application at anthesis.

451   4.2.   Influences of the weather conditions during grain development

452   on yield and protein concentration

453 Weather conditions strongly affect grain yield as well as grain quality of wheat

454 (Johansson and Svensson, 1998; Schulz et al., 2015). The higher grain yield in 2016

455 can be explained by the lower temperature during grain development, which slows

456 down the process of senescence and therefore extends the phase of grain filling.

457 Johansson and Svensson (1998) observed a positive correlation between the hours

458 of sunshine in May and June and the GPC as well as a negative correlation between

459 the amount of rainfall in June and the GPC. These findings are supported by the

460 results of our experiment which show less GPC in consequence of fewer hours of

461 sunshine and more rainfall during grain development (Figure 1 and Figure 3).

462   4.3.   Thousand grain weight and Hagberg falling number as

463   influenced by N fertiliser management

464 Grain yield is composed of parameters, such as seed rate, shoots per plant and

465 grain size. TGW was increased by foliar N application at anthesis but there was no

466 effect of foliar N application on grain yield (Figure 4 and Figure 1). It can be

467 concluded that due to the foliar N application the wheat plants produced fewer but

468 larger grains. Demotes-Mainard et al. (1999) found that the number of grains is

469 usually determined before anthesis, which contradicts the conclusion indicated

470 above. During sampling in the field, there were more sterile florets observed in plots

471   which had received a foliar N application. This could be the reason for the impact of

472   foliar N application on TGW even if the number of florets (potential grains) has been

473   determined earlier. The foliar N application at anthesis only improved HFN at the low

474   N fertilisation level (N100). This finding is consistent with the statement of Gooding

475   and Davies (1992) that foliar application of urea can reduce α-amylase activity and

476   therefore improve HFN.

477   4.4.   Grain protein composition as influenced by N fertiliser

478   management

479   The composition of grain protein determines the end-use quality of wheat flours and

480   was analysed in samples from year 2016.

481   The results of SDS-PAGE show an increased amount of HMW-GS due to the foliar N

482   application at anthesis in two of the three tested varieties (Skyfall and Soissons). The

483   amounts of LMW-GS and α-/β-/γ-gliadins were also positively affected by the foliar N

484   application (Figure 6 A). The ω-gliadins were the only group that remained

485   unaffected by the application of foliar N. Daniel and Triboi (2000) and Hurkman et al.

486   (2013) also report an increase of HMW-GS in response to N fertilisation but they

487   found that ω-gliadins increase as well and amounts of LMW-GS decrease.

488   Considering the results from the SE-HPLC analysis, it is noteworthy that even

489   though the N fertiliser level increased GPC, the effect on the composition of grain

490   proteins was rather small. The N fertiliser level only affected the amounts of the

491   fractions F2 (LMW-GS) and F4 (α-/γ-gliadins) but these changes caused a significant

492   ($p < 0.05$) influence of N fertiliser level on both total gliadins and total glutenins (Figure

493   6 B). This confirms the results from Xue et al., 2016b and Fuertes-Mendizábal et al.,

494   2010, who also reported extended amounts of gliadins and glutenins due to a higher

495   N fertilisation rate. The amounts of gliadins and glutenins were also influenced by an

496   additional foliar N application at anthesis. Since both protein fractions were enlarged

497   by 21 % as a result of a foliar N application, the ratio of gliadins to glutenins

498   remained unchanged (Figure 7 C). Triboï et al. (2000) also observed equal increases

499   of gliadins and glutenins in response to N supply and therefore, no changes in their

500   ratio. On the basis of protein composition modelling, Martre et al. (2006) suggest a

501   transcriptional regulation of the accumulation of protein fractions.

502   4.5.   Concentration and composition of grain protein and their

503   connection to bread volume

504   Grain N concentration was enhanced by N level as well as foliar N application

505   (Figure 1 B). As the bread volume only increased due to a foliar N application at a

506   low N fertilisation rate (N100), it can be concluded that an increasing grain protein

507   concentration does not necessarily lead to an improved baking quality. Baking

508   performance rather depends on the composition of grain proteins. According to

509   Triboi et al. (2003), the storage protein composition strongly depends on the quantity

510   of N in the grain, which is in contrast to the present findings, as increased GPCs due

511   to a foliar N application at a high N fertilisation level (N200) did not lead to a further

512   improvement of baking quality. Millar (2003) states that bread-making varieties

513   generally tend to have higher HMW-GS/LMW-GS and lower gliadin/HMW-GS

514   values. However, the results of our experiment show the opposite effect. At a low N

515   fertiliser level (N100), the foliar N application at anthesis decreased the HMW-

516   GS/LMW-GS ratio and increased the gliadin/HMW-GS ratio. These alterations led to

517   an improved bread volume. Interestingly, the foliar N effect on the ratios of HMW-

518   GS/LMW-GS and gliadin/HMW-GS only occurred when N fertiliser supply was low

519   (N100). There was no such effect observed at high N fertilisation level. The changes

520   in bread volume follow the same pattern: bread volume was increased by foliar N

521   application at low N fertiliser level (N100) but not when N supply was high (N200).

522   The increased GPC due to foliar N application at the high N fertilisation level did not

523   lead to further improvements in bread volume.

524   Surprisingly, the baking performance of the bread-making variety Skyfall was

525   comparable to that of the feed wheat variety Conqueror. A possible explanation for

526   the poor bread volume of Skyfall could lie in the flour type used for the micro-scale

527   baking test. As wholemeal flour was used for the baking tests and Skyfall is a variety

528   designed for the British market, where there is mainly bread consumed which is

529   produced from white flour, this might have particularly affected the results of Skyfall.

530   It can be speculated that Skyfall is unsuitable for being processed in the form of

531   wholemeal flour but might show a good baking performance when used in form of

532   white flour.

533   The parameter which could explain variance in bread volume in our experiment best

534   was the total amount of gliadins measured by SE-HPLC (r^2=0.718). Grain protein

535   concentration only accounted for 60 % of the variance in bread volume when all

536   three varieties were considered. When regression analysis was performed

537   separately for the varieties, the correlation was weakest for Soissons (r^2=0.575)

538   which had the highest GPC and strongest for Conqueror (r^2=0.747) which had the

539   lowest GPC. This finding confirms the weaker relationship between GPC and bread

540   volume in high protein varieties discovered by Gabriel et al. (2017). This

541   demonstrates that the evaluation of bread making quality of wheat flour should not

542   depend on GPC alone but needs consideration of storage protein composition as

543   well.

544

5. Conclusion

545

546 In this study, an increasing GPC did not necessarily generate an improved baking

547 quality, as the increased GPC due to foliar N application at the high N fertilisation

548 level did not lead to further improvements in bread volume. Baking performance

549 rather depends on the composition of grain proteins. At a low N fertiliser level

550 (N100), the foliar N application at anthesis decreased the HMW-GS/LMW-GS ratio

551 and increased the gliadin/HMW-GS ratio, which led to an improved bread volume.

552 No such effects were observed at high N fertilisation level (N200). In this study, the

553 parameter which could explain variance in bread volume best was the total amount

554 of gliadins.

555 These results imply that a late foliar application of nitrogen can be used to effectively

556 improve baking quality in case the total N uptake was low due to unfavourable

557 weather conditions during growing season until anthesis. The findings also show that

558 this effect cannot be observed by measuring GPC alone but also needs a combined

559 inquiry of storage protein composition as well as bread volume. For the future, a

560 quickly detectable parameter for precise bread making quality predictions still needs

561 to be developed. One possibility is that this parameter should be based on the total

562 amount of gliadins.

563

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689 Supplementary information

690 Table 1: Grain yield, grain protein concentration and N harvest index of the three wheat cultivars Conqueror (CN), Soissons (SS)

691 and Skyfall (SY) as affected by N fertilisation (N100 and N200) and foliar N application (+). SD shows the standard deviation of

692 replicates.

Year	Cultivar	Treatment	Grain Yield (t ha^{-1})	SD	GPC (%)	SD	N HI (%)	SD
	CN	N100	7.67	±0.33	6.56	±0.14	79.25	±0.45
		N100+	8.45	±0.29	7.78	±0.11	80.66	±0.33
		N200	10.82	±0.13	8.85	±0.19	79.44	±0.40
		N200+	10.45	±0.44	9.84	±0.14	80.17	±1.41
2016	SS	N100	6.51	±0.24	8.00	±0.09	81.38	±2.26
		N100+	6.90	±0.88	9.70	±0.24	82.26	±0.79
		N200	9.24	±0.18	10.52	±0.19	77.99	±1.22
		N200+	10.49	±0.28	11.87	±0.27	78.25	±0.92
	SY	N100	6.34	±0.19	7.40	±0.35	80.02	±0.50
		N100+	7.82	±1.55	8.46	±0.56	80.45	±1.28
		N200	9.60	±1.34	9.72	±0.38	80.73	±0.44
		N200+	10.43	±0.52	11.11	±0.11	80.63	±0.52
	CN	N100	7.44	±1.00	6.91	±0.24	81.97	±0.57
		N100+	8.11	±1.25	8.50	±0.26	82.25	±0.63
		N200	9.65	±0.36	9.79	±0.33	82.75	±1.12
		N200+	8.92	±0.31	11.18	±0.03	81.33	±1.07
2017	SS	N100	5.40	±1.39	8.85	±0.45	85.10	±0.24
		N100+	5.78	±1.19	10.52	±0.87	84.31	±1.56
		N200	4.40	±0.97	12.09	±0.70	85.67	±0.71
		N200+	5.88	±2.14	13.47	±0.61	85.47	±1.10

SY	N100	6.89	±0.76	7.94	±0.21	84.02	±1.08
	N100+	7.53	±0.65	9.24	±0.20	84.58	±0.37
	N200	9.95	±0.67	10.63	±0.12	84.80	±0.31
	N200+	9.28	±0.71	12.02	±0.30	84.12	±0.64

694 Table 2: Thousand grain weight (TGW) and Hagberg falling number (HFN) of the

695 three wheat cultivars Conqueror (CN), Soissons (SS) and Skyfall (SY) as affected by

696 N fertilisation level (N100 and N200) and foliar N application (+). SD shows the

697 standard deviation of replicates.

Cultivar	Treatment	TGW (g)	SD	HFN (s)	SD
CN	N100	43.87	±0.35	137.67	±23.03
	N100+	47.33	±0.75	233.33	±28.92
	N200	42.83	±1.03	263.33	±11.72
	N200+	44.10	±0.95	251.33	±60.10
SS	N100	35.97	±1.17	297.00	±27.47
	N100+	41.43	±0.31	317.00	±43.31
	N200	36.63	±0.72	327.67	±5.03
	N200+	36.83	±0.64	330.67	±3.06
SY	N100	44.57	±1.07	299.00	±44.80
	N100+	48.43	±0.15	355.67	±31.01
	N200	45.50	±1.37	362.00	±13.08
	N200+	47.23	±0.86	386.00	±10.39

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