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3 **Impact of late-season N fertilisation strategies on the**
4 **gluten content and composition of high protein wheat**
5 **grown under humid Mediterranean conditions**

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15 **Abstract**

16 The rise in high protein common wheat in humid Mediterranean areas has determined a
17 need to compare specific and effective nitrogen (N) fertilisation protocols in order to increase
18 their end-use value. The aim of the work was to assess the impact of late-season N
19 fertilisation strategies on grain yield and protein content (GPC), gluten fraction composition,
20 and rheological traits. Different applications and types of fertiliser (soil applied ammonium
21 nitrate, soil applied urea, foliar applied urea and a foliar applied commercial fertiliser) were
22 distributed at the same rate (30 kg N ha⁻¹) in a field experiment in NW Italy, during three
23 growing seasons. A control without any late-season N fertilisation was also considered. All
24 the treatments received 130 kg N ha⁻¹ as ammonium nitrate (AN), which was split between
25 tillering and the beginning of the stem elongation growth stages.

26 None of the compared late-season N fertilisations significantly affected canopy greenness
27 and stay green duration during the grain filling period, or the grain yield, test weight, and
28 thousand kernel weight, although the foliar application significantly increased foliage burning
29 (+9.8%). The late application of N consistently increased GPC (+1.1%) and dough strength
30 (W, +21%) in the different growing seasons. The type of fertilisation strategies clearly
31 affected the gluten content and rheological parameters: AN was more effective than urea as
32 a soil top-dressed applied fertiliser in increasing W (+10%), as a result of a higher rise in the
33 GPC content (+0.5%) and extensibility (L, +11%). The foliar application at anthesis, at the
34 same N rate, led to a comparable GPC and W with those of the soil top-dressed granular
35 fertiliser. Only a weak effect of granular urea on y/x type HMW was observed for the gluten
36 composition. Conversely, a notable influence of year was observed (i.e. GS/Glia and y/x
37 type HMW), which in turn resulted in a significant impact on W and P and on the aggregation
38 time and aggregation energy.

39 This study offers a further contribution to the improvement of specific N fertilisation strategies
40 in order to enhance the wheat quality according to its end-use value.

41 **Keywords**

42 improver wheat; foliar N fertilisation; gluten proteins; flour quality.

43

44 **Abbreviations**

45 AN, ammonium nitrate; ANOVA, analysis of variance; AUCGC, area under canopy
46 greenness curve; BE, Brabender equivalent; GDDs, growing degree days; GliA, gliadins;
47 GS, glutenins; GPC, Grain protein content; GPE, GlutoPeak equivalent; HMW-GS, high
48 molecular weight glutenins; LMW-GS, low molecular weight glutenins; N, nitrogen; MALDI-
49 TOF/MS Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; P/L,
50 tenacity/extensibility ratio; PMT, peak maximum time; TW, test weight; TKW, thousand
51 kernel weight; W, dough strength.

52

53

54 **1. Introduction**

55 In the last decade, the volatility of prices, the increasing quality needs of the milling and food
56 sector, together with the necessity of guaranteeing a higher income for cereal farmers, have
57 led to a more careful search for quality, according to the specific end use of wheat. High
58 protein ($> 14\%$) and high dough strength ($W > 350 \text{ J} \cdot 10^{-4}$) are desirable for high protein
59 common wheat, which is classified as improver wheat (in Italy), excellent or class E wheat
60 (in France and Germany), or Hard Red wheat (in the U.S.A), according to the specific
61 classification terminology of the different countries.

62 The quality of wheat mainly depends on the variety and on its ability to accumulate protein
63 reserves in the grain and the management of nitrogen (N) fertilisation (Hellemans et al.,
64 2018). Thus, the specialization of these high protein wheat categories requires the need of
65 specific and effective N fertilisation protocols to increase their end-use value in different
66 growing areas, in particular in humid Mediterranean areas where their cultivation is
67 increasing. Several studies have reported that the distribution of late-season N (30 – 50 kg
68 N ha^{-1}) – in addition to a sufficient N application in the vegetative growth stages - is an
69 essential practice to achieve the quality requests of these wheat categories in climatic zones
70 with adequate spring rainfall or in irrigated cropping systems (Brown and Petrie, 2006). Late-
71 season N fertilisation may be carried out through the application of a top-dressed soil
72 granular fertiliser between the booting and the heading growth stages or through the
73 frequent spraying of concentrated foliar N fertiliser in mixtures with fungicides at flowering,
74 in order to control Fusarium head blight (FHB) and other foliar disease (Woolfolk et al.,
75 2002).

76 Late-season foliar N applications, compared to soil applications, could provide such benefits
77 as a reduction in N losses through denitrification and leaching, a more rapid utilization of
78 nutrients and an increased ability to make N available when the root activity is impaired.
79 Furthermore, as far as the leaf burn is concerned, there is a maximum N rate that can be

80 distributed without resulting in damage (Brown and Petrie, 2006; Woolfolk et al., 2002),
81 particularly in temperate areas with high temperatures during application. Leaf burning, as
82 a result of foliar urea applications, is mainly related to the biuret content, which is toxic for
83 crops (Mikkelsen, 1990). However, foliar N applications at a low rate ($<10 \text{ kg N ha}^{-1}$), in order
84 to avoid leaf burning, resulted in a less effective and stable protein quality enhancement
85 than a higher application rate of top-dressed soil granular fertiliser (Blandino et al., 2015).
86 The studies carried out until now have mainly focused on the effect of the late season N rate
87 and the application timing. Since the availability of N between flowering and the end of the
88 ripening is the main requirement for the accumulation of proteins in the grain, the form of
89 application and the type of N fertiliser could also play important roles. Moreover, the rapidity
90 of the N fertiliser of providing the nutritive element to the crop is related above all to the
91 source of N: urea requires more time than ammonium fertilisers, whereas nitrate is rapidly
92 effective (Recous et al., 1998). In addition, recent studies on the effect on the gluten content
93 (Xue et al., 2016a; Zhong et al., 2019) have also highlighted how a late N application could
94 also contribute to varying the composition of the protein reserves (gluten). However, the
95 recently acquired information is somewhat contradictory and the investigation of late-season
96 N strategy fertilisation on agronomical, productive, rheological traits and on the gluten
97 content and quality requires a deeper field study, in which the complex interaction with
98 environmental conditions should be taken into account. The aim of the work was to analyse
99 the impact of late-season N fertilisation strategies, taking into consideration different
100 applications, different types of fertiliser, at the same rate, on grain yield, protein content,
101 gluten quality and the composition of gluten fractions in high protein common wheat. The
102 objective was to make a further contribution to the development of specific and effective
103 fertilization strategies for humid Mediterranean areas in order to enhance the quality of high
104 protein wheat cultivars.

105

106 **2. Material and methods**

107 **2.1. Experimental site and treatments**

108 The study was carried out on the North-West Italian plain at Carmagnola (44° 50' N, 7° 40'
109 E; elevation 245 m), over three growing seasons (2013-14, 2014-15 and 2015-16). The daily
110 temperatures and precipitation were measured at a meteorological station near the
111 experimental area. The main soil information is reported in Table S1. The experiment was
112 performed on the experimental farm of the University of Turin in a deep silty-loam soil (Typic
113 Udifluvents), characterised by a medium cation-exchange capacity and organic matter
114 content.

115 Four different late-season N fertilisation strategies were compared with a control without late
116 N application (untreated control):

- 117 ▪ Soil applied ammonium nitrate (AN), 30 kg N ha⁻¹ was top-dressed applied as solid
118 prills (27% N w/w) at the beginning of heading (growth stage 52, Zadoks et al., 1974);
- 119 ▪ Soil applied urea, 30 kg N ha⁻¹ was top-dressed applied as solid prills (46% N w/w)
120 at the beginning of heading (growth stage 52);
- 121 ▪ Foliar applied urea, 30 kg N ha⁻¹ was foliar applied at anthesis (growth stage 65)
122 using solid prill urea (46% N w/w, biuret content of 1% w/w) previously dissolved in
123 water to obtain an aqueous solution;
- 124 ▪ Foliar applied fertiliser, 30 kg N ha⁻¹ was foliar applied at anthesis (growth stage 65)
125 using the Folur® liquid commercial fertiliser (Tradecorp International, Madrid, Spain,
126 soluble liquid, composition 22% ureic N w/w and a low biuret content < 0.05% w/w).

127 A total of 130 kg N ha⁻¹ was top-dressed applied in all the treatments as a granular
128 ammonium nitrate fertiliser, split as 50 kg N ha⁻¹ at tillering (growth stage 23) and 80 kg N
129 ha⁻¹ at the beginning of stem elongation (growth stage 32). The list of treatments and details
130 of the N fertilisation are summarised in Table S2.

131 All the top-dressed granular fertilisers were applied by hand, while the leaf N fertilisers were
132 distributed using a precision plot sprayer.

133 The main experimental information for each growing season is reported in Table S3. The
134 cultivar was Rebelde (Apsovsementi, Voghera, Italy), which is classified as improver winter
135 common wheat, because of its high protein content.

136 The treatments were assigned to experimental units using a completely randomised block
137 design with four replicates. The plot size was 7 x 1.5 m.

138 All the trials were treated with a fungicide at wheat anthesis using a mixture of prothioconazole
139 and tebuconazole (Prosaro[®], Bayer, Italy) applied at 0.125 kg + 0.125 kg of active ingredient
140 (AI) ha⁻¹ at flowering (growth stage 65) to avoid Fusarium Head Blight infection and to protect
141 against flag leaf greenness.

142

143 **2.2. Canopy greenness during ripening**

144 A hand-held optical sensing device, GreenSeekerTM[®] (Trimble, Sunnyvale, California, the
145 USA), was used to measure the normalized difference vegetation index (NDVI) from
146 flowering to the end of the grain filling stage. The instrument was held approximately 80 cm
147 above the canopy and its effective spatial resolution was 2 m².

148 The NDVI values were proportional to the crop biomass and greenness. The Area Under
149 Canopy Greenness Curve (AUCGC) was calculated during grain filling for each treatment,
150 starting from the NDVI measurement for each observation date and using the following
151 formula:

$$152 \text{ AUCGC} = \sum_i^{n-1} \{[(R_i + R_{i+1})/2] (t_{i+1} - t_i)\}$$

153 where R is the NDVI value, t is the time of observation and n is the number of observations
154 (6).

155

156

157 **2.3. Foliar burn severity**

158 The severity of foliage burning, linked to the late-season N application, was evaluated on
159 the leaves at the soft dough stage (growth stage 85) in each plot. Leaf burning was classified
160 in 7 classes (0 = 0%; 1 = 2.5%; 2 = 5%; 3 = 10%; 4 = 25%; 5 = 50%; 6 = > 50%), according
161 to the visible symptoms. Fifteen randomly selected flag leaves and 15 penultimate leaves
162 were considered for each plot.

163

164 **2.4. Grain yield**

165 The grain yields were obtained by harvesting the whole plot with a Walter Wintersteiger
166 cereal plot combine-harvester. Grain moisture was analysed using a Dickey-John GAC2100
167 grain analyser (Auburn, IL, USA). The grain yield results were adjusted to a 13% moisture
168 content. The harvested grains were mixed thoroughly and 4 kg grain samples were taken
169 from each plot for the qualitative analyses.

170

171 **2.5. Kernel quality traits**

172 The test weight (TW), thousand kernel weight (TKW) and grain protein content (GPC;
173 Kjeldahl N x 5.7, on a dry matter basis) were determined according to Blandino et al. (2015).

174

175 **2.6. Rheological properties**

176 Grains (3 kg) from each plot was milled using the Bona 4RB mill (Bona, Monza, Italy) in
177 order to obtain refined flour.

178 The alveograph test was carried out on the refined flour according to ICC-121 (ICC, 1992).

179 Gluten aggregation properties were measured using GlutoPeak (Brabender GmbH and Co
180 KG, Duisburg, Germany), according to the method reported by Marti et al. (2015). Briefly,
181 flour (9 g) was dispersed in distilled water (10 ml). During the test, the sample and water

182 temperature were maintained at 35 °C by circulating water through the jacketed sample cup.
183 The paddle was set to rotate at 3000 rpm and each test was run for 500 s. Curves were
184 elaborated using the software provided with the instrument (Brabender GlutoPeak v 2.1.2)
185 and the following indices were considered: i) Maximum Torque, expressed in Brabender
186 Equivalentents (BE) - corresponding to the peak that occurs when gluten aggregates; ii) Peak
187 Maximum Time (PMT), expressed in seconds, which corresponds to the peak torque time;
188 iii) aggregation energy, expressed as the GlutoPeak Equivalent (GPE), which corresponds
189 to the area under the portion of the curve 15s before and 5 s after the peak. The test was
190 carried out for the 2014-15 and 2015-16 growing seasons and data related to the control
191 and soil applied AN samples were shown. The test was carried out in duplicate on three
192 different plots per treatment.

193

194 **2.7. Gluten protein quantification**

195 Gliadins, HMW-GS and LMW-GS were extracted from refined flour using a previously
196 reported sequential extraction procedure (Visioli et al., 2017). The relative protein
197 quantification was determined by means of a colorimetric Bradford assay (Biorad Hercules,
198 CA). Three biological replicates were performed for each sample. The extracted fractions
199 were then dried in a Savant SpeedVac SPD1010 device (Thermo Fisher Scientific,
200 Walthman,MA, the USA) at 45°C and were then utilised for SDS-PAGE. Exact masses of
201 the members of each gluten fraction were also obtained in MALDI-TOF/MS analysis linear
202 mode, as previously described (Visioli et al., 2017).

203

204 **2.8. Gliadin and glutenin separation by means of SDS-PAGE and densitometry** 205 **analyses**

206 SDS-PAGE was performed in a Mini-PROTEAN Tetra Cell (Bio-Rad) on 7.5% and 12%
207 acrylamide gel for the HMW-GS, the LMW-GS and the gliadin fractions, respectively. An

208 aliquot of 2.5 µg of dried HMW-GS and 7 µg of LMW-GS and gliadins was suspended in 20
209 µL of loading buffer containing 2% (w/v) SDS, 0.02% (w/v) bromophenol blue, 0.1% β-
210 mercaptoethanol, 0.05 M Tris-HCl pH 6.8 and 10% (v/v) glycerol, and boiled at 95 °C for 5
211 min before loading onto the gel. A ColorBurst™ High Range Marker Electrophoresis device
212 (Mw 30,000-220,000) was used to detect HMW-GS, and a Molecular-Weight Marker® (Mw
213 14,000-66,000; Sigma Aldrich, St. Louis, MO, the USA) was used to detect the LMW-GS
214 and gliadins. After electrophoretic separation at 40 mA, the gels were stained with a brilliant
215 blue G-colloidal solution (Sigma Aldrich) fixed in 7% (v/v) acetic acid and 40% (v/v)
216 methanol, and de-stained in 25% (v/v) methanol (Figure S1). The HMW-GS, LMW-GS and
217 gliadins were analysed in three technical replicates for each plot sample. IMAGE lab 4.5.1
218 (Bio-Rad) software was used for the relative quantification of the gliadin, LMW-GS and
219 HMW-GS single protein sub-units on each gel.

220

221 **2.9. Statistical analysis**

222 The Kolmogorov–Smirnov normality test and the Levene test have been carried out to verify
223 the normal distribution and homogeneity of variances. All the productive and rheological
224 parameters were compared by means of an analysis of variance (ANOVA), in which the late-
225 season N fertilisation and the year were the independent variables. An ANOVA was used to
226 compare the relative abundances of the gluten fractions, in which a combination of the
227 different late-season N fertilisations and the year were the independent variables. Multiple
228 comparison tests were performed according to the Ryan-Einot-Gabriel-Welsh F (REGW-F)
229 test on treatment means. Statistical data analysis was carried out with the SPSS software
230 package, version 24.0.

231 **3. Results**

232 **3.1. Weather conditions**

233 In the period between wheat sowing (November) and flowering (May) different rainfall were
234 recorded in the three growing seasons (Table S4): the 2014-15 growing season resulted in
235 the greatest total amount of rainfall (> 850 mm), while the precipitation in the vegetative
236 stage of 2015 -16 was inferior than 300 mm. The different frequencies and intensities of
237 rainfall during the winter resulted in a different availability of N in the soil, with the lowest and
238 highest values in the 2014-15 and 2015-16 growing seasons, respectively. The 2013 – 14
239 growing season was characterised by the lowest rainfall during ripening (May – June). The
240 growing season with the highest GDD was the 2014 –15 season, in particular from April to
241 June, and this lead to an accelerated crop senescence.

242

243 **3.2. Agronomical and productive parameters**

244 The late-season N fertilisation had a clear impact on foliar burn severity (Table 1). A
245 negligible leaf burn was recorded in the untreated control and for the granular top-dressed
246 fertilisations, while the foliar application significantly increased foliage burning, generally at
247 the flag leaf apex. The burn severity was increased by 8.3% and 11.2% for the foliar fertiliser
248 (low biuret content) and foliar urea dissolved in water, respectively. The average foliar burn
249 severity was not significantly different for the considered growing seasons, and the
250 interaction was never significant.

251 The late-season N fertilisation did not affect the AUCGC to a great extent during the grain
252 filling period, or the grain yield, test weight and TKW. As far as the year effect is concerned,
253 2015-16 showed the highest AUCGC index value as a consequence of a better distribution
254 of rainfall during the growing season and resulted in a significantly higher grain yield
255 (+12.4%) than the other considered growing seasons. The kernels harvested in the driest
256 2013-14 growing season resulted in a significantly lower test weight, while the lowest

257 thousand kernel weight was registered for the 2014-15 growing season. The interactions
258 with the years were never significant for any of the productive parameters.

259

260 **3.3. Protein content and gluten composition**

261 The late application of 30 kg N ha⁻¹ significantly increased the GPC for all of the compared
262 N fertilisation strategies (Table 2). Furthermore, when applied at the same rate, the soil
263 applied urea resulted in a significantly lower enhancement of the protein concentration in
264 the grain than the ammonium nitrate top-dressed distribution. On average, the late
265 application of granular ammonium nitrate and urea led to an increase in GPC of 1.1% and
266 0.6%, respectively. Both of the N foliar applications at flowering resulted in a similar
267 enhancement of the protein concentration to that recorded for the soil distribution of
268 ammonium nitrate. The 2014-15 growing season resulted in a significantly higher GPC than
269 the other compared growing seasons, while the interaction was never significant.

270 Although the same wheat cultivar and the same field were considered in each experiment,
271 a notable difference in the gluten composition was recorded for the compared years. The
272 relative proportions of different gliadin and glutenin subunits and their ratio were analysed
273 according to the fertilisation practices and years (Table 2; Figures 1, 2, 3). Different gluten
274 sub-units were clearly and significantly influenced by the crop season. However, a significant
275 effect of late-season N fertilisation was only observed for the y/x type HMW-GS, while the
276 late N application had no impact on the GS/Glia and HMW/LMW-GS ratio.

277 The HMW-GS x-type (low in S-bonds) showed a decrease in abundance from 2013-14 to
278 the following growing seasons, while the HMW-GS y-type (rich in S-bonds) showed an
279 opposite trend (Figure 1). As a result, the y/x type HMW-GS ratio decreased clearly from the
280 2015-16 period to the 2014-15 and 2013-14 seasons, in agreement with the AUCGC values
281 (Table 2). In addition, the application of urea, either as top-dressed soil or foliar applied, led
282 to a significant increase in the y/x type HMW-GS, while the effect of late applications of

283 ammonium nitrate or the commercial liquid fertiliser was not significantly different from the
284 control (Table 2).

285 As far as LMW-GS are concerned, the LMW-GS 39 kDa was the most abundant in all the
286 treatments and for all the growing seasons, with a significant increase in 2014-15 compared
287 to the other two crop seasons. The same trend was observed for the LMW-GS 36 kDa, while
288 a slight decrease in LMW-GS 32 kDa and 31kDa were instead observed in 2014-15 (Figure
289 2).

290 As for the gliadin fractions, S-poor fractions with a molecular weight of 55-39 kDa (ω -gliadins
291 enriched fraction) were significantly more abundant in the 2013-14 season than in the
292 following crop years; S-rich fractions with a molecular weight of 35-31kDa (γ -gliadins
293 enriched fraction) were significantly more abundant in the 2014-15 season than in the other
294 years, and reached the lowest value in 2015-16, while the opposite trend was observed for
295 the 35-28 kDa molecular weight gliadins ($\alpha\beta$ -gliadin enriched fraction), with a significant
296 higher abundance in the 2015-16 season (Figure 3).

297 The N treatments did not affect the amounts of gliadins or the LMW-GS fractions. The
298 different responses of the γ and $\alpha\beta$ -gliadin enriched fractions on the application of urea as
299 top-dressed soil, with respect to the other treatments, was only related to the 2013-14
300 season.

301 The 2014-15 growing season, which was characterized by the lowest TKW and the highest
302 protein content, showed the highest GS/Glia and HMW/LMW-GS ratio. The 2015-16 year,
303 with the highest AUCGC and grain yield, showed the lowest GS/Glia, but the highest y/x
304 type HMW-GS ratio. The interaction was never significant for any of the previously reported
305 gluten fraction ratios.

306

307

308

309 **3.4. Rheological properties**

310 The late-season N fertilisation strategy significantly affected dough strength (W),
311 extensibility (L) and P/L ratio, while no differences were observed for dough tenacity (P)
312 (Table 2). The fertilisation strategies led to different impacts, in terms of dough strength.
313 Specifically, compared to the control, the increase in W was +26%, +15%, +22%, +19% for
314 the soil applied ammonium nitrate, soil applied urea, foliar applied urea and foliar applied
315 commercial fertiliser, respectively. The use of top-dressed ammonium nitrate instead of urea
316 significantly increased the W by 10%.

317 The highest P/L value was recorded for the control without late-season N fertilisation, while
318 only the top-dressed soil application of ammonium nitrate was able to significantly reduce
319 the value of this parameter, through the achievement of the highest value of L. Moreover,
320 the other fertilisation strategies led to a significant increase in L, compared to the control,
321 although the increase was more contained than that observed for the use of ammonium
322 nitrate.

323 As far as gluten aggregation kinetics is concerned, the late-season N fertilisation significantly
324 decreased the time required for the maximum aggregation (PMT) to be reached, and
325 significantly increased both the maximum consistency and the aggregation energy (Table 3;
326 Figure S2). Overall, the results confirm a clear positive effect of late season N fertilisation
327 on gluten strength. As far as the effect of year is concerned, the 2015-16 growing season
328 resulted in the highest W, P/L and P values as well as in the longest PMT and in the highest
329 aggregation energy, but also the lowest L value. Conversely, the growing season did not
330 significantly affect the maximum consistency, as measured by the GlutoPeak test.

331 The interaction was never significant for the parameters obtained from either the Alveograph
332 or the GlutoPeak test.

333 **4. Discussion**

334 The pooled data on the influence of an increased temperature and CO₂ concentration and
335 the modification in rainfall distribution should result in a clear reduction in the wheat protein
336 concentration (Asseng et al., 2019). This negative effect is particularly important for high
337 protein common wheat, whose optimum end-use quality and market price are closely related
338 to the protein content and to the related rheological traits. In this context, the study of a more
339 efficient use of N fertilisers becomes more crucial to promote the accumulation of proteins
340 in the grain and to investigate the potential impact on protein functionality. In order to avoid
341 a negative environmental impact of N-pollutants and to establish the most efficient use of
342 this input, it seems more interesting to comprehend the possible effect of the fertilisation
343 strategies and form than of the application of a higher N rate.

344

345 **4.1. Effect of late-season N form and timing**

346 This study shows that a late season N application in temperate Mediterranean growing
347 areas, with a medium-short interval between anthesis and plant senescence (30-45 days),
348 has no impact on the duration of grain filling (AUCGC), the grain yield or the yield
349 parameters, such as test weight and TKW. Conversely, a clear effect of late season N
350 application on GPC and dough strength (W) has been confirmed. On average, the
351 application of 30 kg N ha⁻¹ as ammonium nitrate at heading is responsible for a +1.1% and
352 + 91 J*10⁻⁴ enhancement of GPC and W, respectively. In a 6-year experiment (Blandino et
353 al., 2016), carried out in the same growing area, but with a less performing cultivar, in terms
354 of grain protein accumulation, the increase in GPC and W, as a result of the application of
355 40 kg N ha⁻¹ at booting, was +1.1% and +76 J * 10⁻⁴, respectively. Improvements in GPC
356 (between 1.0% and 1.3%) have also been shown for late-season N applications of between
357 27 and 56 kg N ha⁻¹ (Brown and Petrie, 2006; Dick et al., 2016). The positive effect of late
358 fertilisation on protein quantity does not seem to be due to the higher N rate than the control,

359 but to the late growth stage N splitting, without any additional N fertilisation input (Xue et al.,
360 2016a; 2016b). Moreover, delaying the timing of the N application closer to crop anthesis
361 also has a positive impact on the physiological remobilisation of the stored N, as a result of
362 a higher protein degradation and N transport from the leaves to the grains during grain filling
363 (Zhong et al., 2018), but also a more efficient use of the N sources in the soil (Fuertes-
364 Mendizábal et al., 2018).

365 In addition to the general role of late-season N application, the current study also highlights
366 an effect of both mode and form of N distribution on the rheological quality of high protein
367 wheat. As far as the granular soil distribution is concerned, AN salt resulted in a significantly
368 more pronounced effect on GPC and W enhancement than urea. The rise in W is related to
369 an increase in dough extensibility (L), while no effect on dough tenacity (P) was reported for
370 any of the compared fertilisation strategies. In the present study, the soil distribution of AN
371 resulted in a clearly greater increase in L than urea. As far as the dough handling properties
372 are concerned, the increase in L is positive for many improver wheat cultivars, since it
373 contributes to equilibrating their unbalanced P/L ratio, which is often characterised by an
374 excessive tenacity (Sanchez-Garcia et al., 2015). Similar results were obtained when AN
375 and urea were used as the only fertiliser applied to wheat over the whole crop cycle
376 (Sylvester-Bradley et al., 2014). At wheat booting, ammonium nitrate or nitrate-N resulted in
377 a greater increase in gluten content and bread volume than urea (Xue et al., 2016a; 2016b).
378 The different effects on GPC, according to the forms of N (AN vs urea), could be related to
379 the higher volatilisation losses of urea compared to AN (Sylvester-Bradley et al., 2014),
380 particularly for late-season applications during spring when the temperatures are higher.
381 Thus, in order to avoid ammonia volatilization, urea should be applied with urease inhibitors
382 or as soil injections.

383 Moreover, focusing on post anthesis N acquisition, Bogard et al. (2011) reported that the N
384 availability at anthesis is more important than the duration of senescence during grain filling.

385 Thus, considering the late-season distribution of the present experiment, the quicker
386 availability of AN applied at the beginning of wheat heading, compared to the slow
387 solubilisation of urea, could result in a more efficient uptake and translocation of this element
388 to the grain. Xue et al. (2016b) also highlighted the importance of the timing of late-season
389 fertilisation, and thus of the N uptake timing, on GPC; the N recovery in grain was lower
390 when N was applied at heading rather than at booting. The positive prompt N availability of
391 AN could be more important in the considered growing area, with a duration of the
392 phenological phases lower than that of other environments, such as Northern Europe.
393 Since post anthesis N uptake depends on the occurrence of adequate soil moisture content
394 that is able to favor its absorption by plant roots, a foliar N application may be an interesting
395 substitute strategy to enhance the GPC content under dry conditions, that could occur under
396 humid Mediterranean conditions. Moreover, a foliar N distribution can be used together with
397 a fungicide at wheat flowering, and this would result in a lower cost than a granular top-
398 dressed soil distribution, which requires a dedicated passage (Blandino et al. 2015). In
399 previous experiments carried out in the same environment (Blandino et al., 2015; 2016),
400 applying a foliar N fertiliser at a minor rate led to the leaves being kept safe from any leaf
401 burns (5 kg N ha^{-1}) but also to a less effective and stable GPC quality enhancement than
402 the top-dressed soil distribution of a granular fertiliser (40 kg N ha^{-1}). However, the present
403 study, in a humid Mediterranean growing area, has highlighted that a foliar application, when
404 compared at the same rate of N, could lead to a comparable GPC and flour strength to those
405 of the granular soil top-dressed fertiliser, thus confirming results obtained also in North
406 Europe by Gooding et al. (2007). It is interesting to note that the same fertiliser form (urea)
407 resulted in a higher GPC enhancement when the foliar fertilisation was applied at anthesis
408 than a soil top-dressed distribution at heading. However, no differences have been observed
409 for the GPC and alveographic parameters for the compared ureic foliar strategies.
410 Furthermore, the high rate of foliar ureic application resulted in significant leaf burn, in

411 particular when conventional urea was used, and although no negative impact on AUCGC
412 and grain yield have been recorded, the visible effect on the crop during ripening could
413 discourage farmers from adopting this solution. In order to reduce this negative effect, it may
414 be necessary to split the application into two timings, applying the fertiliser in the coolest
415 hours of the day and using the products with a low biuret content (Woolfolk et al., 2002).

416 Despite the clear differences in the GPC, under the considered conditions, the late-season
417 N application did not visibly influence the gluten composition. With the exception of the effect
418 of urea application on the y/x type HMW, in comparison to the control, no significant
419 variations in the GS/glia or H/L ratio, or any clear modification of the percentages of the
420 gluten subunits have been recorded for the fertilisations with different N forms and strategies

421 The proportions of gluten proteins have been shown to change according to the N
422 application rate: the S-poor gluten fractions, ω -gliadins and HMW-GS were often increased
423 to a great extent by higher levels of N supply than S-rich gliadin and LMW-GS (Hurkman et
424 al., 2013). In a pot experiment, the distribution of an additional N rate at a late booting stage
425 significantly increased the relative abundance of Glia and of the x-type HMW-GS (Xue et
426 al., 2016a). In the study of Wieser and Seilmeier (1998), a further late-season N fertilisation
427 (80 kg N ha^{-1}) increased the Glia/GS, HMW/LMW-GS and the y-type/x-type HMW-GS ratio,
428 compared to the control. Rossmann et al. (2019) reported that late-season foliar application
429 of urea (40 kg N ha^{-1}) clearly increase GPC of ordinary bread-making cultivars, although
430 only at low N fertilizer level this treatment decreased the HMW/LMW and the Glia/HMW
431 ratio. Moreover, Rekowski et al. (2019) highlighted in a pot experiment that the variation of
432 gluten fraction as a consequence of late season N application could varied according to the
433 wheat cultivars.

434 As far as the type of fertiliser is concerned, contrasting results have been reported in
435 literature. In the study of Fuertes-Mendizábal et al. (2013), the distribution of ammonium as
436 the only N source during the whole plant development period increased the GPC and

437 affected the GS/Glia ratio compared to a nitrate-N source. On the other hand, the type of
438 late-season N fertiliser (granular ammonium nitrate or urea) did not affect the Glia/GS or the
439 HMW/LMW-GS ratio, in either a pot experiment (Xue et al., 2016a) or in an open field
440 experiment (Xue et al., 2016b). In the case of durum wheat, no differences in the total gluten,
441 Glia/GS or HMW/LMW-GS ratio were observed for soil (urea) and foliar (urea ammonium
442 nitrate) treatments applied at heading, whereas the method of application was observed to
443 have influenced the proportion of certain gliadin classes (in the 39-30 KDa range) and the
444 most abundant LMW-GS subunits with a variety dependent effect (Visioli et al., 2017).
445 The low impact of post-anthesis N acquisition on the total grain N might account for the lack
446 of influence of the late N application form and strategies on the gluten composition. In fact,
447 the majority of grain N (>75%) originates from a remobilisation from the canopy rather than
448 from post-anthesis uptake (Li et al., 2016). Changes in gluten composition, as a
449 consequence of the fertiliser supply (fertiliser form, timing, method), may be possible with a
450 higher proportion of late-season applications or in wheat with a low N rate at stem
451 elongation. Moreover, since both N uptake after anthesis and N remobilisation depend on
452 the senescence process, a late-season N supply could determine an effect on the gluten
453 composition, if it leads to differences in the senescence process in environments with a
454 longer duration of the filling period.

455

456 **4.2. Effect of environmental growth conditions**

457 Unlike the N fertilisation strategies, the meteorological conditions were found to affect the
458 gluten composition to a great extent, and this resulted in clear differences in the rheological
459 properties, which were measured with either conventional (i.e. Alveograph) or new (i.e.
460 GlutoPeak) approaches. The 2013-14 and 2014-15 seasons showed similar trends, with the
461 latter showing a high GPC, as a consequence of the low TKW. Although the highest GPC,
462 GS/Glia and HMW/LMW-GS ratios were observed for the 2014-15 growing season, the flour

463 did not result in the highest *W* for the considered growing seasons. The GS, and its insoluble
464 fraction (namely glutenin macropolymer, GMP) content, are generally correlated to dough
465 strength (Thanhaeuser et al., 2014). The 2015-16 season was different from the other two
466 seasons as far as the gluten protein class content and ratios are concerned, and a lower
467 GS/Glia ratio was observed as a consequence of the high gliadin content accumulated in
468 the grains, related to the longer ripening period (highest value of AUCGC). In addition, even
469 though the relative content of HMW-GS for the three years was similar, there was a
470 significant increase in HMW-GS γ -type in 2015-16, and this resulted in a higher y/x ratio of
471 the HMW-GS components. This index could be correlated with the higher alveographic *P*
472 value obtained in the 2015-16 season, since the HMW-GS γ -type presents 6 Cys residues
473 in its sequence while the α -type presents 4 Cys residues, which could allow more inter chain
474 S-S bonds and result in a stronger gluten matrix. In addition, the lower *L* value could be due
475 to the decrease in the relative amounts of γ -gliadin enriched fraction with respect to $\alpha\beta$ -
476 gliadin enriched fractions. The role of the different classes of gliadins in the characteristics
477 of dough is still under investigation. Both $\alpha\beta$ - and γ -gliadins have the possibility of interacting
478 more with gluten because of their high number of Cys residues respect to ω -gliadins which
479 are Cys poor proteins (Barak et al., 2015). Despite the similar number of Cys residues, $\alpha\beta$ -
480 gliadins adopt a globular protein structure, while γ -gliadins have extended and rod-like
481 structures, which could determine the extensibility characteristics of dough (Ang et al.,
482 2010).

483 As a result of the changes in the gluten profiles, the growing year (i.e. weather conditions)
484 affected the dough rheology to a great extent. Dough tenacity (*P*) was not affected by the
485 fertilisation strategies considered in this study, while its high value was responsible for the
486 extremely high *P/L* ratio in the flours from the 2015-16 growing season (Table 6).
487 Furthermore, the N application late in the growing season, particularly when AN is used,

488 lead to a rise of L values, resulting in a positive reduction of P/L in genotype or growing
489 areas with high dough tenacity.

490 In the last part of the study, the effect of late season N fertilisation on gluten quality for the
491 years with the greatest difference in gluten composition (2014-15 vs 2015-16) was assessed
492 using a new rapid high shear-based approach, i.e. the GlutoPeak test. The increase in
493 maximum torque and aggregation energy when late N was applied is in agreement with the
494 increase in GPC and alveographic indices, suggesting an increase in dough strength (Marti
495 et al., 2015). The wheat flours from the 2015-16 growing season showed higher aggregation
496 energy – thus resulting in a higher strength - than the flours from the 2014-15 growing
497 season, which is in accordance with the W and P indices and the content of the y/x type.
498 The dramatically high P/L of the 2015-16 dough is in agreement with the gluten aggregation
499 kinetics measured by the GlutoPeak test. Indeed, these samples are characterized by a
500 wide peak, long PMT and high aggregation energy, likely due to the high levels of the HMW-
501 GS y-type, which are rich in S-bonds.

502 **5. Conclusions**

503 The increasing interest in the production of common wheat with specific quality traits
504 requires the development of fertilisation strategies that are able to guarantee a higher
505 constancy of the desired rheological parameters. Although the late N fertilisation in the
506 considered growing area had no impact on the agronomical traits or grain yield, this practice
507 consistently increased GPC and dough strength in different growing seasons. The type of
508 fertilisation strategies clearly affects the gluten content and rheological parameters: AN is
509 more effective than urea in increasing W with the soil top-dressed applied fertilizer as a
510 result of a greater rise in the GPC content and L. The latter effect may contribute positively
511 towards reducing P/L in cultivars and environments characterised by excessive dough
512 tenacity. The foliar application applied at anthesis, at the same rate, could lead to a
513 comparable GPC and W to that of the soil top-dressed granular fertiliser.

514 Moreover, the late-season N application, at the applied N rate, despite the clear differences
515 in the GPC, did not influence the gluten composition (the relative ratio of the percentage of
516 gluten subunits and of the gluten fraction). Conversely, the data collected for the same
517 cultivar, field and agronomic management practices over a 3-year period reported a notable
518 influence of the meteorological conditions on the gluten composition, and greater differences
519 in the rheological properties were observed. In addition to W, the year of cultivation also
520 showed a great impact on the P/L ratio, and P was affected more than L. The rise in P/L is
521 linked to the reduction in GS/Glia in the growing season with the longest ripening period,
522 accompanied by an increase in the y/x type HMW-GS.

523 In short, the late-season N fertilisation of improver wheat positively enhanced dough
524 strength through an increase in GPC and dough extensibility, and the type of applied
525 fertilisation strategies influenced these effects. Moreover, the application on an N fertiliser
526 close to wheat ripening did not seem to have any impact on the gluten composition, while

527 the growing season played a key role in affecting the relative ratio between the gluten
528 subunits and consequently both the dough strength and tenacity.

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629

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