Journal of Cereal Science xxx (xxxx) xxx-xxx

Contents lists available at ScienceDirect

Journal of Cereal Science

journal homepage: http://ees.elsevier.com

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

Massimo Blandino[a,*], Giovanna Visioli[b], Silvia Marando[b], Alessandra Marti[c], Amedeo Reyneri[a]

[a] Università degli Studi di Torino, Department of Agricultural, Forest and Food Science, Grugliasco(TO), Italy
[b] Università degli Studi di Parma, Department of Chemistry, Life Sciences and Environmental Sustainability, Parma, Italy
[c] Università degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences, Milan, Italy

ARTICLE INFO

Keywords
Improver wheat
Foliar N fertilisation
Gluten proteins
Flour quality

ABSTRACT

The rise in high protein common wheat in humid Mediterranean areas has determined a need to compare specific and effective nitrogen (N) fertilisation protocols in order to increase their end-use value. The aim of the work was to assess the impact of late-season N fertilisation strategies on grain yield and protein content (GPC), gluten fraction composition, and rheological traits. Different applications and types of fertiliser (soil applied ammonium nitrate, soil applied urea, foliar applied urea and a foliar applied commercial fertiliser) were distributed at the same rate (30 kg N ha^{-1}) in a field experiment in NW Italy, during three growing seasons. A control without any late-season N fertilisation was also considered. All the treatments received 130 kg N ha^{-1} as ammonium nitrate (AN), which was split between tillering and the beginning of the stem elongation growth stages.None of the compared late-season N fertilisations significantly affected canopy greenness and stay green duration during the grain filling period, or the grain yield, test weight, and thousand kernel weight, although the foliar application significantly increased foliage burning (+9.8%). The late application of N consistently increased GPC (+1.1%) and dough strength (W, +21%) in the different growing seasons. The type of fertilisation strategies clearly affected the gluten content and rheological parameters: AN was more effective than urea as a soil top-dressed applied fertiliser in increasing W (+10%), as a result of a higher rise in the GPC content (+0.5%) and extensibility (L, +11%). The foliar application at anthesis, at the same N rate, led to a comparable GPC and W with those of the soil top-dressed granular fertiliser. Only a weak effect of granular urea on y/x type HMW was observed for the gluten composition. Conversely, a notable influence of year was observed (i.e. GS/Glia and y/x type HMW), which in turn resulted in a significant impact on W and P and on the aggregation time and aggregation energy.This study offers a further contribution to the improvement of specific N fertilisation strategies in order to enhance the wheat quality according to its end-use value.

Abbreviations

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

1. Introduction

In the last decade, the volatility of prices, the increasing quality needs of the milling and food sector, together with the necessity of guaranteeing a higher income for cereal farmers, have led to a

* Corresponding author.
E-mail address: massimo.blandino@unito.it (M. Blandino)

https://doi.org/10.1016/j.jcs.2020.102995
Received 20 January 2020; Received in revised form 7 May 2020; Accepted 7 May 2020
Available online xxx
0733-5210/© 2020.

more careful search for quality, according to the specific end use of wheat. High protein (>14%) and high dough strength (W > 350 J*10^{-4}) are desirable for high protein common wheat, which is classified as improver wheat (in Italy), excellent or class E wheat (in France and Germany), or Hard Red wheat (in the U.S.A), according to the specific classification terminology of the different countries.

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

Late-season foliar N applications, compared to soil applications, could provide such benefits as a reduction in N losses through denitrification and leaching, a more rapid utilization of nutrients and an increased ability to make N available when the root activity is impaired. Furthermore, as far as the leaf burn is concerned, there is a maximum N rate that can be distributed without resulting in damage (Brown and Petrie, 2006; Woolfolk et al., 2002), particularly in temperate areas with high temperatures during application. Leaf burning, as a result of foliar urea applications, is mainly related to the biuret content, which is toxic for crops (Mikkelsen, 1990). However, foliar N applications at a low rate (<10 kg N ha^{-1}), in order to avoid leaf burning, resulted in a less effective and stable protein quality enhancement than a higher application rate of top-dressed soil granular fertiliser (Blandino et al., 2015). The studies carried out until now have mainly focused on the effect of the late season N rate and the application timing. Since the availability of N between flowering and the end of the ripening is the main requirement for the accumulation of proteins in the grain, the form of application and the type of N fertiliser could also play important roles. Moreover, the rapidity of the N fertiliser of providing the nutritive element to the crop is related above all to the source of N: urea requires more time than ammonium fertilisers, whereas nitrate is rapidly effective (Recous et al., 1988). In addition, recent studies on the effect on the gluten content (Xue et al., 2016a; Zhong et al., 2019) have also highlighted how a late N application could also contribute to varying the composition of the protein reserves (gluten). However, the recently acquired information is somewhat contradictory and the investigation of late-season N strategy fertilisation on agronomical, productive, rheological traits and on the gluten content and quality requires a deeper field study, in which the complex interaction with environmental conditions should be taken into account. The aim of the work was to analyse the impact of late-season N fertilisation strategies, taking into consideration different applications, different types of fertiliser, at the same rate, on grain yield, protein content, gluten quality and the composition of gluten fractions in high protein common wheat. The objective was to make a further contribution to the development of specific and effective fertilisation strategies for humid Mediterranean areas in order to enhance the quality of high protein wheat cultivars.

2. Material and methods

2.1. Experimental site and treatments

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

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

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

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

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

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

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

All the trials were treated with a fungicide at wheat anthesis using a mixture of protioconazole and tebuconazole (Prosaro®, Bayer, Italy) applied at 0.125 kg + 0.125 kg of active ingredient (AI) ha^{-1} at flowering (growth stage 65) to avoid Fusarium Head Blight infection and to protect against flag leaf greenness.

2.2. Canopy greenness during ripening

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

The NDVI values were proportional to the crop biomass and greenness. The Area Under Canopy Greenness Curve (AUCGC) was calculated during grain filling for each treatment, starting from the NDVI measurement for each observation date and using the following formula:where R is the NDVI value, t is the time of observation and n is the number of observations (6).

2.3. Foliar burn severity

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

2.4. Grain yield

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

2.5. Kernel quality traits

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

2.6. Rheological properties

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

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

Gluten aggregation properties were measured using GlutoPeak (Brabender GmbH and Co KG, Duisburg, Germany), according to the method reported by Marti et al. (2015). Briefly, flour (9 g) was dispersed in distilled water (10 ml). During the test, the sample and water temperature were maintained at 35 °C by circulating water through the jacketed sample cup. The paddle was set to rotate at 3000 rpm and each test was run for 500 s. Curves were elaborated using the software provided with the instrument (Brabender GlutoPeak v 2.1.2) and the following indices were considered: i) Maximum Torque, expressed in Brabender Equivalents (BE) - corresponding to the peak that occurs when gluten aggregates; ii) Peak Maximum Time (PMT), expressed in seconds, which corresponds to the peak torque time; iii) aggregation energy, expressed as the GlutoPeak Equivalent (GPE), which corresponds to the area under the portion of the curve 15s before and 5 s after the peak. The test was carried out for the 2014-15 and 2015-16 growing seasons and data related to the control and soil applied AN samples were shown. The test was carried out in duplicate on three different plots per treatment.

2.7. Gluten protein quantification

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

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

SDS-PAGE was performed in a Mini-PROTEAN Tetra Cell (Bio-Rad) on 7.5% and 12% acrylamide gel for the HMW-GS, the LMW-GS and the gliadin fractions, respectively. An aliquot of 2.5 μg of dried HMW-GS and 7 μg of LMW-GS and gliadins was suspended in 20 μL of loading buffer containing 2% (w/v) SDS, 0.02% (w/v) bromophenol blue, 0.1% β-mercaptoethanol, 0.05 M Tris-HCl pH 6.8 and 10% (v/v) glycerol, and boiled at 95 °C for 5 min before loading onto the gel. A ColorBurst™ High Range Marker Electrophoresis device (Mw 30,000–220,000) was used to detect HMW-GS, and a Molecular-Weight Marker® (Mw 14,000–66,000; Sigma Aldrich, St. Louis, MO, the USA) was used to detect the LMW-GS and gliadins. After electrophoretic separation at 40 mA, the gels were stained with a brilliant blue G-colloidal solution (Sigma Aldrich) fixed in 7% (v/v) acetic acid and 40% (v/v) methanol, and de-stained in 25% (v/v) methanol (Fig. S1). The HMW-GS, LMW-GS and gliadins were analysed in three technical replicates for each plot sample. IMAGE lab 4.5.1 (Bio-Rad) software was used for the relative quantification of the gliadin, LMW-GS and HMW-GS single protein sub-units on each gel.

2.9. Statistical analysis

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

3. Results

3.1. Weather conditions

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

3.2. Agronomical and productive parameters

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

Table 1
Effect of late season N fertilisation strategies on foliar burn severity, area under the canopy greenness curve (AUCCG) during the grain filling and productive parameters in 2013–2016 period in North Italy.

Factor	Source of **variation**	Foliar burn severity		AUCGC		Grain yield		Test weight		Thousand kernel weight	
		%				t ha $^{-1}$		kg hl $^{-1}$		g	
Late-season N fertilisation	untreated control	0.3	c	25.7	a	6.1	a	83.2	a	34.6	a
	soil applied AN	0.3	c	26.0	a	6.4	a	83.2	a	35.0	a
	soil applied urea	0.4	c	25.8	a	6.1	a	83.2	a	35.5	a
	foliar applied urea	11.5	a	25.5	a	6.3	a	83.1	a	34.8	a
	foliar applied fertiliser	8.6	b	25.7	a	6.0	a	83.2	a	35.9	a
	P level	< 0.001		0.537		0.490		0.995		0.06	
Year	2013–14	4.1	a	23.3	c	5.8	b	81.4	b	35.9	a
	2014–15	4.7	a	25.5	b	6.1	b	84.0	a	33.4	b
	2015–16	3.9	a	28.3	a	6.7	a	83.9	a	36.3	a
	P level	0.107		< 0.001		< 0.001		< 0.001		< 0.001	
N x Year	P level	0.480		0.963		0.553		0.635		0.144	

Means followed by different letters are significantly different (the level of significance P is shown in the table).
The reported values for the late season N fertilisation are based on 12 replications (3 years X 4 repetitions), while the values per year are based on 20 replications (5 late season N fertilisation X 4 repetitions).

The late-season N fertilisation did not affect the AUCGC to a great extent during the grain filling period, or the grain yield, test weight and TKW. As far as the year effect is concerned, 2015-16 showed the highest AUCGC index value as a consequence of a better distribution of rainfall during the growing season and resulted in a significantly higher grain yield (+12.4%) than the other considered growing seasons. The kernels harvested in the driest 2013-14 growing season resulted in a significantly lower test weight, while the lowest thousand kernel weight was registered for the 2014-15 growing season. The interactions with the years were never significant for any of the productive parameters.

3.3. Protein content and gluten composition

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

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

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

Table 2
Effect of late season N fertilisation strategies on grain protein content (GPC), glutenin/gliadin (GS/Glia) ratio, high molecular weight/low molecular weight (H/L) glutenins, y/x type ratio of high molecular weight glutenin and alveographic parameters in 2013–2016 period in North Italy.

Factor	Source of variation	GPC		gluten fraction ratio						alveographic parameters							
				GS/glia		H/L (GS)		y/x type (H)		W		P/L		P		L	
		%								J * 10 $^{-4}$				mm		mm	
Late-season N fertilisation	untreated control	14.5	c	1.0	a	0.85	a	0.62	b	349	c	2.1	a	122	a	60	c
	soil applied AN	15.6	a	1.1	a	0.79	a	0.69	ab	440	a	1.6	b	127	a	78	a
	soil applied Urea	15.1	b	1.0	a	0.80	a	0.75	a	401	b	1.9	ab	127	a	70	b
	foliar applied Urea	15.5	a	1.0	a	0.78	a	0.80	a	424	ab	1.8	ab	128	a	73	ab
	foliar applied fertiliser	15.7	a	1.1	a	0.84	a	0.69	ab	416	ab	1.8	ab	127	a	70	b
	P level	< 0.001		0.425		0.252		0.028		< 0.001		0.004		0.237		< 0.001	
Year	2013–14	15.0	b	1.1	b	0.76	b	0.44	c	406	b	1.7	b	123	b	73	a
	2014–15	15.7	a	1.3	a	0.87	a	0.64	b	358	c	1.6	b	111	c	71	a
	2015–16	15.1	b	0.8	c	0.81	b	1.00	a	451	a	2.3	a	146	a	66	b
	P level	< 0.001		< 0.001		0.002		< 0.001		< 0.001		< 0.001		< 0.001		0.004	
N x Year	P level	0.832		< 0.001		0.177		0.137		0.963		0.202		0.471		0.427	

Means followed by different letters are significantly different (the level of significance P is shown in the table).
The reported values for the late season N fertilisation are based on 12 replications (3 years X 4 repetitions), while the values per year are based on 20 replications (5 late season N fertilisation X 4 repetitions).

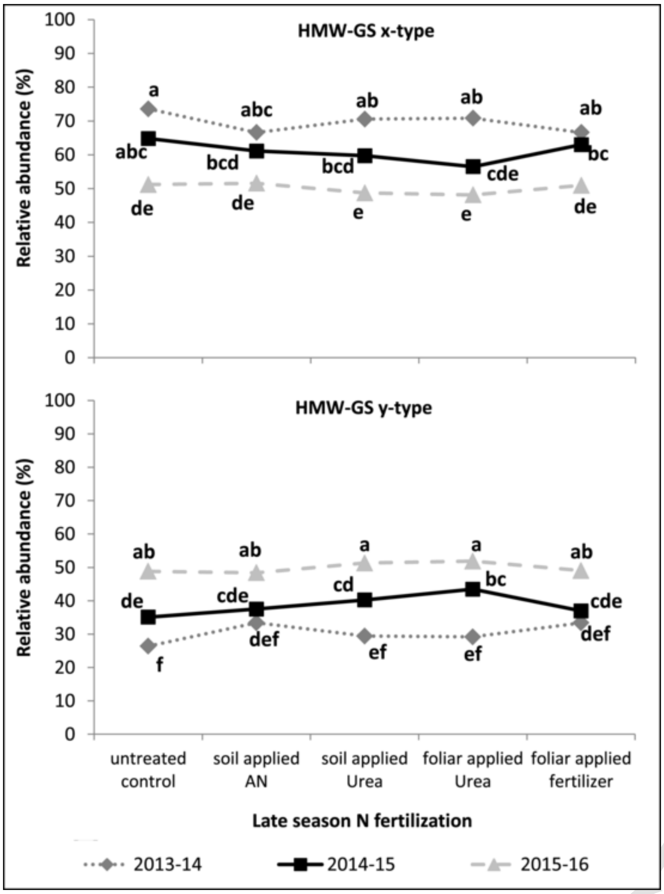

Fig. 1. Relative abundances of the x- and y-types of high molecular weight glutenins in different late season N fertilisation strategies and 3 growing seasons obtained from densitometric analysis.The data are the means of three replicates. Means followed by different letters are significantly different ($P < 0.05$).

in the y/x type HMW-GS, while the effect of late applications of ammonium nitrate or the commercial liquid fertiliser was not significantly different from the control (Table 2).

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

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

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

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

3.4. Rheological properties

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

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

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

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

4. Discussion

The pooled data on the influence of an increased temperature and CO_2 concentration and the modification in rainfall distribution should result in a clear reduction in the wheat protein concentration (Asseng et al., 2019). This negative effect is particularly important for high protein common wheat, whose optimum end-use quality and market price are closely related to the protein content and to the related rheological traits. In this context, the study of a more efficient use of N fertilisers becomes more crucial to promote the accumulation of proteins in the grain and to investigate the potential impact on protein functionality. In order to avoid a negative environmental impact of N-pollutants and to establish the most efficient use of this input, it seems more interesting to comprehend the possible effect of the fertilisation strategies and form than of the application of a higher N rate.

4.1. Effect of late-season N form and timing

This study shows that a late season N application in temperate Mediterranean growing areas, with a medium-short interval between anthesis and plant senescence (30–45 days), has no impact on the duration of grain filling (AUCGC), the grain yield or the yield parameters, such as test weight and TKW. Conversely, a clear effect of late season N application on GPC and dough strength (W) has been confirmed. On average, the application of 30 kg N ha^{-1} as ammonium nitrate at heading is responsible for a +1.1% and +91 $J*10^{-4}$ enhancement of GPC and W, respectively. In a 6-year experiment (Blandino et al., 2016), carried out in the same growing area, but with a less performing cultivar, in terms of grain protein accumulation, the increase in GPC and W, as a result of the application of 40 kg N ha^{-1} at booting, was +1.1% and +76 $J * 10^{-4}$, respectively. Improvements in GPC (between 1.0% and 1.3%) have also been shown for late-season N applications of between 27 and 56 kg N ha^{-1} (Brown and Petrie, 2006; Dick et

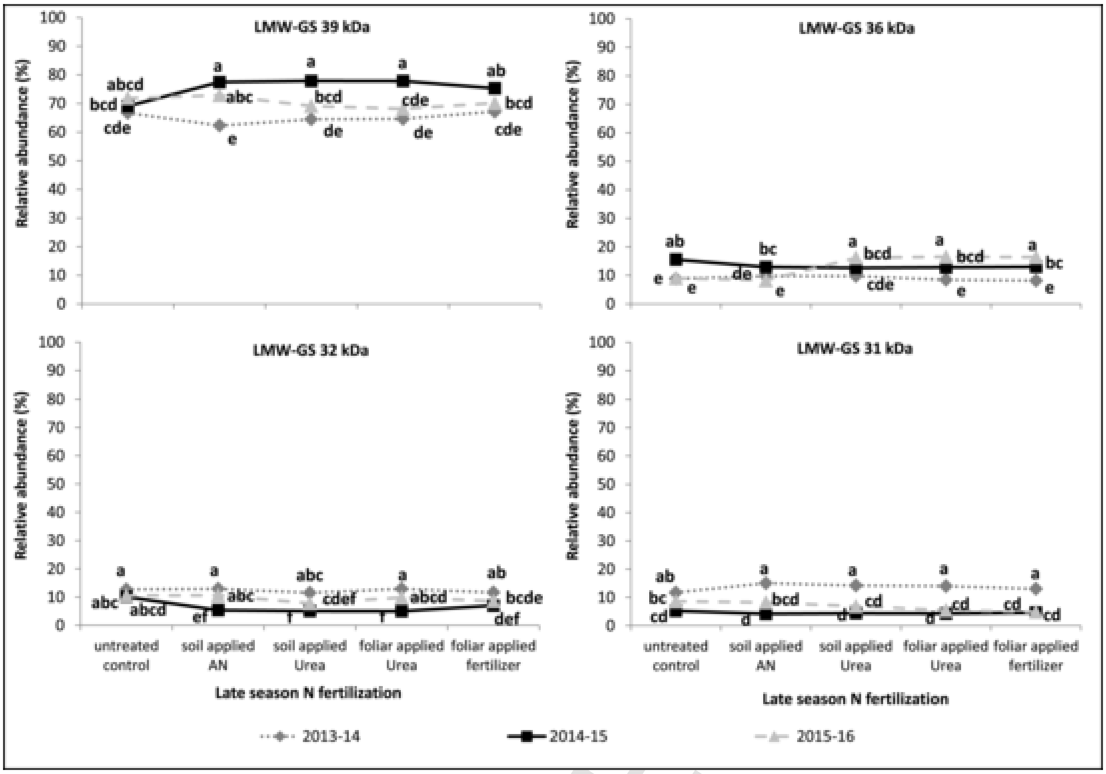

Fig. 2. Relative abundances of the low molecular weight glutenins for different late season N fertilisation strategies and 3 growing seasons obtained from densitometric analysis.The data are the means of three replicates. Means followed by different letters are significantly different ($P < 0.05$).

al., 2016). The positive effect of late fertilisation on protein quantity does not seem to be due to the higher N rate than the control, but to the late growth stage N splitting, without any additional N fertilisation input (Xue et al., 2016a, 2016b). Moreover, delaying the timing of the N application closer to crop anthesis also has a positive impact on the physiological remobilisation of the stored N, as a result of a higher protein degradation and N transport from the leaves to the grains during grain filling (Zhong et al., 2018), but also a more efficient use of the N sources in the soil (Fuertes-Mendizábal et al., 2018).

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

Moreover, focusing on post anthesis N acquisition, Bogard et al. (2011) reported that the N availability at anthesis is more important than the duration of senescence during grain filling. Thus, considering the late-season distribution of the present experiment, the quicker availability of AN applied at the beginning of wheat heading, compared to the slow solubilisation of urea, could result in a more efficient uptake and translocation of this element to the grain. Xue et al. (2016b) also highlighted the importance of the timing of late-season fertilisation, and thus of the N uptake timing, on GPC; the N recovery in grain was lower when N was applied at heading rather than at booting. The positive prompt N availability of AN could be more important in the considered growing area, with a duration of the phenological phases lower than that of other environments, such as Northern Europe.

Since post anthesis N uptake depends on the occurrence of adequate soil moisture content that is able to favor its absorption by plant roots, a foliar N application may be an interesting substitute strategy to enhance the GPC content under dry conditions, that could occur under humid Mediterranean conditions. Moreover, a foliar N distribution can be used together with a fungicide at wheat flowering, and this would result in a lower cost than a granular top-dressed soil distribution, which requires a dedicated passage (Blandino et al., 2015). In previous experiments carried out in the same environment (Blandino et al., 2015, 2016), applying a foliar N fertiliser at a minor rate led to the leaves being kept safe from any leaf burns (5 kg N ha^{-1}) but also to a less effective and stable GPC quality enhancement than the top-dressed soil distribution of a granular fertiliser (40 kg N ha^{-1}). However, the present study, in a humid Mediterranean growing area, has highlighted that a foliar application, when compared at the same rate of N, could lead to a comparable GPC and flour strength to those of the granular soil top-dressed fertiliser, thus confirming results obtained also in North Europe by Gooding et al. (2007). It is interesting to note that the same fertiliser form (urea) resulted in a higher GPC enhancement when the foliar fertilisation was applied at anthesis than a soil top-dressed distribution at heading. However, no differences have been observed for the GPC and alveographic parameters for the compared ureic foliar strategies. Furthermore, the high rate of foliar ureic application re-

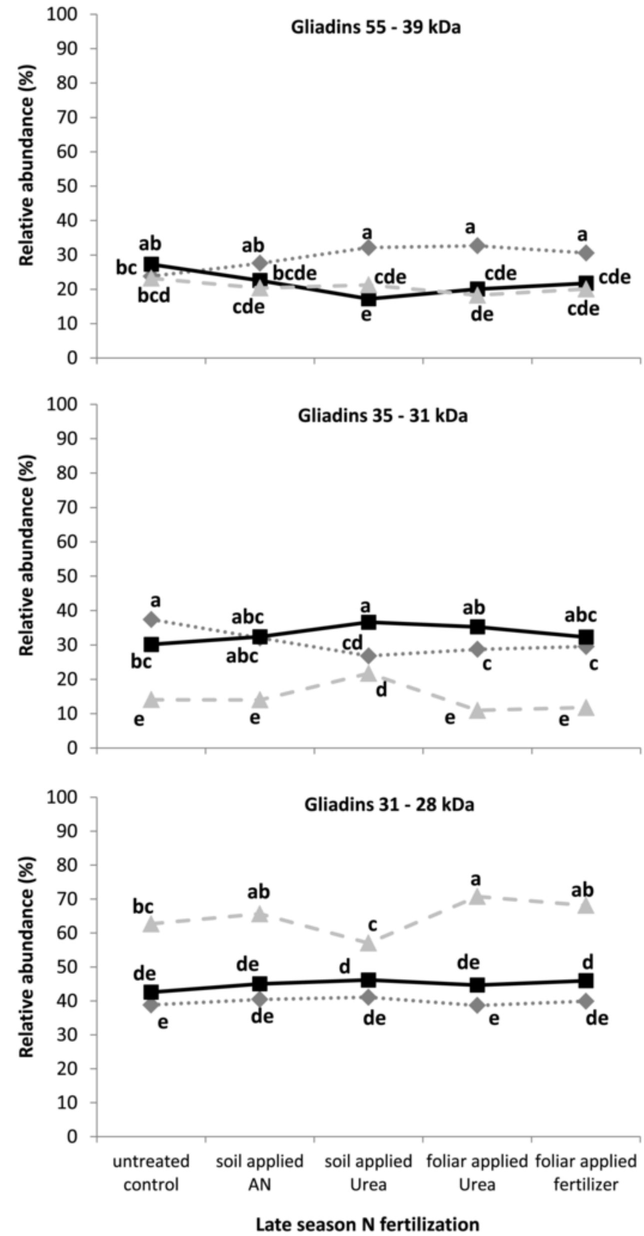

Fig. 3. Relative abundances of the gliadin enriched fractions for different late season N fertilisation strategies and 3 growing seasons obtained from densitometric analysis.The data are the means of three replicates. Means followed by different letters are significantly different (P < 0.05).

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

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

Table 3
Effect of late season N fertilisation strategies and year on gluten aggregation properties.

Factor	Source of variation	Peak maximum		Maximum		Aggregation	
		Time (PMT)		torque		Energy	
		s		BE		GPE	
Late-season N fertilisation	untreated control	303	a	52	b	1298	b
	soil applied AN	267	b	56	a	1417	a
	P level	0.050		0.021		0.010	
Year	2014–15	256	b	54	a	1301	b
	2015–16	314	a	54	a	1414	a
	P level	0.006		0.700		0.013	
N x Year	P level	0.754		0.760		0.386	

Means followed by different letters are significantly different (the level of significance P is shown in the table).
The reported values for the late season N fertilisation are based on 6 replications (2 years X 3 repetitions), while the values per year are based on 6 replications (2 late season N fertilisations X 3 repetitions).

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

As far as the type of fertiliser is concerned, contrasting results have been reported in literature. In the study of Fuertes-Mendizábal et al. (2013), the distribution of ammonium as the only N source during the whole plant development period increased the GPC and affected the GS/Glia ratio compared to a nitrate-N source. On the other hand, the type of late-season N fertiliser (granular ammonium nitrate or urea) did not affect the Glia/GS or the HMW/LMW-GS ratio, in either a pot experiment (Xue et al., 2016a) or in an open field experiment (Xue et al., 2016b). In the case of durum wheat, no differences in the total gluten, Glia/GS or HMW/LMW-GS ratio were observed for soil (urea) and foliar (urea ammonium nitrate) treatments applied at heading, whereas the method of application was observed to have influenced the proportion of certain gliadin classes (in the 39-30 KDa range) and the most abundant LMW-GS subunits with a variety dependent effect (Visioli et al., 2017).

The low impact of post-anthesis N acquisition on the total grain N might account for the lack of influence of the late N application form and strategies on the gluten composition. In fact, the majority of grain N (>75%) originates from a remobilisation from the canopy rather than from post-anthesis uptake (Li et al., 2016). Changes in gluten composition, as a consequence of the fertiliser supply (fertiliser form, timing, method), may be possible with a higher proportion of late-season applications or in wheat with a low N rate at stem elongation. Moreover, since both N uptake after anthesis and N remobilisation depend on the senescence process, a late-season N supply could determine an effect on the gluten composition, if it leads to differences in the senescence process in environments with a longer duration of the filling period.

4.2. Effect of environmental growth conditions

Unlike the N fertilisation strategies, the meteorological conditions were found to affect the gluten composition to a great extent, and this resulted in clear differences in the rheological properties, which were measured with either conventional (i.e. Alveograph) or new (i.e. GlutoPeak) approaches. The 2013-14 and 2014-15 seasons showed similar trends, with the latter showing a high GPC, as a consequence of the low TKW. Although the highest GPC, GS/Glia and HMW/LMW-GS ratios were observed for the 2014-15 growing season, the flour did not result in the highest W for the considered growing seasons. The GS, and its insoluble fraction (namely glutenin macropolymer, GMP) content, are generally correlated to dough strength (Thanhaeuser et al., 2014). The 2015-16 season was different from the other two seasons as far as the gluten protein class content and ratios are concerned, and a lower GS/Glia ratio was observed as a consequence of the high gliadin content accumulated in the grains, related to the longer ripening period (highest value of AUCGC). In addition, even though the relative content of HMW-GS for the three years was similar, there was a significant increase in HMW-GS y-type in 2015–16, and this resulted in a higher y/x ratio of the HMW-GS components. This index could be correlated with the higher alveographic P value obtained in the 2015-16 season, since the HMW-GS y-type presents 6 Cys residues in its sequence while the x-type presents 4 Cys residues, which could allow more inter chain S–S bonds and result in a stronger gluten matrix. In addition, the lower L value could be due to the decrease in the relative amounts of γ-gliadin enriched fraction with respect to αβ-gliadin enriched fractions. The role of the different classes of gliadins in the characteristics of dough is still under investigation. Both αβ- and γ-gliadins have the possibility of interacting more with gluten because of their high number of Cys residues respect to ω-gliadins which are Cys poor proteins (Barak et al., 2015). Despite the similar number of Cys residues, αβ -gliadins adopt a globular protein structure, while γ-gliadins have extended and rod-like structures, which could determine the extensibility characteristics of dough (Ang et al., 2010).

As a result of the changes in the gluten profiles, the growing year (i.e. weather conditions) affected the dough rheology to a great extent. Dough tenacity (P) was not affected by the fertilisation strategies considered in this study, while its high value was responsible for the extremely high P/L ratio in the flours from the 2015-16 growing season (Table 2). Furthermore, the N application late in the growing season, particularly when AN is used, lead to a rise of L values, resulting in a positive reduction of P/L in genotype or growing areas with high dough tenacity.

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

5. Conclusions

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

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

In short, the late-season N fertilisation of improver wheat positively enhanced dough strength through an increase in GPC and dough extensibility, and the type of applied fertilisation strategies influenced these effects. Moreover, the application on an N fertiliser close to wheat ripening did not seem to have any impact on the gluten composition, while the growing season played a key role in affecting the relative ratio between the gluten subunits and consequently both the dough strength and tenacity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank Simona Bellion, Andrea Borio, Raffaella Glave, Federico Marinaccio, Francesca Vanara (Università di Torino), Nelson Marmiroli, Urbana Bonas (Università di Parma), and Gaetano Cardone (Università degli Studi di Milano) for their precious help and cooperation in the laboratory facilities and field work.

The research has been conducted thanks to the financial support of the Cassa di Risparmio di Cuneo (CRC), as a part of the SPECIALWHEAT project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcs.2020.102995.

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