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(Article begins on next page)

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Impact of nitrogen fertilisation strategies on the protein content, gluten composition and rheological properties of wheat for biscuit production

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Abstract

The impact of fertilisation strategies on bread-making quality of wheat has been investigated extensively, while only few studies have been carried out on minor supply-chains, such as the biscuit production chain, whose products require a low protein content (< 10.5%) and weak gluten strength (< 110 J 10^{-4}). The aim of this work was to obtain insight into the effect of N fertilisation strategies on grain yield and quality, by focusing on the changes in the composition of gluten protein and its relationship with dough quality for the biscuit supply-chain. Seven different nitrogen fertilisation strategies, including standard and slow release fertilisers and the combined application of sulphur (S), were compared in relation to the grain yield and quality of two soft wheat cultivars (Artico, Sy Alteo) in a three-years field trial. Canopy development was phenotyped, in terms of NDVI, and nitrogen Agronomic Efficiency was assessed. Rainfall during grain filling influenced the agronomic and quality performances to a great extent. The effect of N fertilisation was more marked on the P/L and gluten composition than on the grain yield and grain protein content. Genotypes showed similar protein content and W values, and greatly differed for P/L. The gluten protein composition analysis showed that P/L was greatly influenced by the proportion of specific glutenin sub-units, both in HMW-GS (Dx2, Bx7) and in LMW-GS (B- and C-type). Sulphur application, with both standard and slow release fertilisers, contributed to reducing the P/L ratio, with an increase in the C-type LMW-GS. Single application of slow release N fertilisers can be recommended for biscuit making to achieve better qualitative requirements, without compromising the grain yield. In addition, the co-application of sulphur with nitrogen showed an increase in grain yield and modulated glutenin composition (B- and C-type LMW-GSs) and P/L with moderate increase of W.

Keywords: gluten composition; nitrogen fertilisation; nitrogen agronomic efficiency; soft wheat; slow release fertiliser

Abbreviations: AUCGC, area under the canopy greenness curve; glia/glut, gliadin-to-glutenin ratio; GDD, growing degree days; GN = number of grain per square meter; GY, grain yield; GPC, grain protein content; GS, growth stage; HMW-GS, high molecular weight glutenin subunits; HMW-GS Dx/Bx, the ratio between Glu-Dx2 and Glu-Bx7 HMW-GSs; H/L, the ratio between HMW-GS and LMW-GS expression; H/L b, the ratio between HMW-GS and the B-type LMW-GS expression; L, dough extensibility; LMW-GS, low molecular weight glutenin subunits; L b/c = ratio between the B-type and C-type LMW-GSs; N, nitrogen; NAE, nitrogen agronomic efficiency; P, dough tenacity; P/L, ratio between dough tenacity and extensibility; S, sulphur; TKW, thousand kernel weight; TW, test weight, W, dough strength.

1. Introduction

The food industry's requirement for homogeneous wheat batches, characterised by a defined end-use, is increasing because of the progressive qualitative specialisation of cereals for the food market and the costumer request of clearer traceability. The grain protein content (GPC) of wheat is the main quality factor that influences its technological properties and then the market classes of this cereal throughout the world. Unlike improved or superior bread-making wheat, which requires a high protein content, a low protein grain is desirable for the soft wheat used for minor supply-chains such as biscuit production (Foca *et al.*, 2007; Farrer *et al.*, 2006). In particular, the good biscuit-making quality of wheat has been defined in terms of a soft kernel texture, a low protein content (<10.5%), a weak (< 110 J 10^{-4}) gluten strength (W) and low alkaline water retention capacity (AWRC) (Gaines, 1991). Moreover, a low ratio (< 0.7 - 0.5) between tenacity and extensibility (P/L) is strongly required to achieve a good biscuit making quality (Labuschagne *et al.*, 1997).

The achievement of the aforementioned qualitative goals mainly depends on the choice of variety; in fact, a key factor to obtaining good biscuit quality wheat is the use of cultivars characterised by high grain softness, which is related to their capacity of accumulating specific storage proteins. Wheat grain proteins consist of soluble metabolic proteins (albumins and globulins) and alcohol-soluble monomeric (gliadin) and polymeric (glutenin) storage proteins, which are also called prolamins; these account for about 70-80% of the total grain protein content. These proteins are classified on the basis of their electrophoretic mobility as ω-, γ- and α-type gliadins and high and low molecular weight glutenin subunits (HMW-GS and LMW-GS, respectively). In common wheat (*Triticum aestivum* L.), a known polymorphism, which is encoded by *Glu-A1*, *Glu-B1* and *Glu-D1* genes, exists in HMW-GS, and it is associated with rheological differences in the final quality (Payne and Lawrence, 1983; Wieser, 2007) which affect the properties of dough (MacRitchie, 2016). The gliadin-to-glutenin ratio might also affect the spread and texture of biscuits; thus, a high spread ratio, the absence of retraction and a uniform thickness are considered criteria of good quality (Barak *et al*., 2013). Igrejas *et al*. (2002) individuated genotypes with 13+16 *Glu-B1* subunits as being particularly suitable for a good end use quality of biscuits. Genotypes with null *Glu-D1* allele have also been found to be suitable (Zhang *et al*., 2018).

In addition to the chosen wheat cultivar, nitrogen (N) fertilisation also influences the content of grain storage proteins to a great extent as well as the consequent rheological quality. A direct relationship exists between the N fertiliser rates and the GPC in wheat (Garrido-Lestache *et al*., 2004; Giuliani *et al*., 2011). Furthermore, high grain protein levels in wheat may in part be attributed to high N application rates, which farmers apply to obtain higher grain yields, often without taking into account luxury consumption (Zorb et al., 2018). In a previous study (Blandino *et al*., 2015), suitable rheological parameters of wheat for biscuits were achieved through a low N rate application at the stem elongation growth stage, but this clearly limited the grain yield performance. Thus, for this wheat category, it is necessary to find an N management compromise between quantitative and qualitative aspects.

The availability of N between anthesis and the end of ripening is the main requirement for the promotion of the accumulation of proteins in the grain, and the distributed dose, but the timing and the form of application, could also play an important role. In temperate winter wheat production areas, N is generally split between tillering (growth stage GS 22-24, according to Zadoks et al., 1974) and the beginning of stem elongation (GS 31-32). Dividing the total N application into two or more treatments can help growers to enhance nutrient efficiency and wheat yields as well as to mitigate the loss of nutrients related to spring precipitations (López-Bellido *et al*., 2005; Giuliani *et al*., 2011). The rapidity of the N fertiliser in providing the nutritive element to the crop is mainly related to the source of N: urea requires more time than ammonium fertilisers, whereas nitrate is rapidly effective (Hawkesford, 2014). The use of special fertilisers, such as slow release or controlled ones, could be an alternative to enhance the efficiency of N fertilisation and to reduce the number of field applications, by minimizing N leaching and denitrification, especially in sandy and shallow soils (Calabi-floody *et al*., 2018). Different solutions and mechanisms to slow the rate of N release are available on the market: fertilisers containing nitrification and urease inhibitors, impermeable or semipermeable coatings which progressively solubilise the granule and organic-mineral fertiliser contents (Weber *et al*., 2009).

In addition, recent studies on the effect of fertilisation on the gluten content (Xue *et al*., 2016; Rekowski *et al*., 2020) have also highlighted how the type of N fertiliser can also contribute to varying the composition of the storage proteins; moreover, the interaction of N fertilisation with the environment can affect the gluten composition (Hurkman *et al*., 2013). Furthermore, also sulphur (S) supply, in association with N fertilisation, has an effect on agronomic performance (Duncan *et al*., 2018); in particular, co-application of N and S is reported to increase N use efficiency and % of N recovery, with a positive effect on protein content (Salvagiotti *et al*., 2009). As for the effect on grain quality, S application is known to improve technological performances, especially on bread and durum wheat (Zhao *et al*., 1999; Ercoli *et al*., 2011). This improvement is mainly due to changes in protein composition, in particular by the increase in the accumulation of sulphur-rich glutenins, HMW-GS and markedly LMW-GS (Pompa *et al*., 2009; Zorb *et al*., 2009; Tao *et al*., 2018). However, while most of the agronomic studies have been conducted in relation to bread-making quality, very little information is available on biscuit cultivars. The aim of this study has been to gain insight into the effect of N fertilisation strategies on grain yield and quality, while focusing on the potential changes in gluten protein content or composition and its relationship with dough quality for the biscuit supply-chain.

2. Materials and Methods

2.1. Experimental site and treatments

The study was carried out in the North-West Po plain in Italy (Cigliano, 45°18' N, 8°01' E, altitude 237 m asl), over three growing seasons (2013/14, 2014/15 and 2015/16). The experiment was performed in a silt loam soil (Typic Hapludalfs according to the USDA classification). According to the common crop practices of the growing areas, the previous crop in each growing season was maize, thus the experiment was carried out on the same farm but using different adjacent fields. The soil was sampled each growing season from 0-30 cm using Eijkelkamp cylindrical augers at wheat tillering (GS23) before the first N fertilisation. The average main physical and chemical properties of the soil for the three crop seasons were: 38.6% sand, 51.8% silt and 9.6% clay; pH 6.2; 1.78% organic matter; C/N ratio 10.8; cation exchange capacity (C.E.C.) 11.5 meq kg^{-1}; 0.096 % total N; 44 mg kg^{-1} available P; 120 mg kg^{-1} exchangeable K.

The compared treatments in each growing season were a factorial combination of:

- two red soft hexaploid wheat cultivars for biscuit production with different alveographic traits: cv. Artico (multiple cross, 2001, Apsovsementi S.p.A., Voghera, PV, Italy) with an equilibrated P/L ratio); cv. Sy Alteo (Innov x Apache, 2010, Syngenta Italia S.p.A., Milan, Italy), generally characterized by high P/L ratio values for this qualitative category.
- seven N granular fertilisation strategies, as detailed in Table 1. Three conventional split fertilisation treatments, applied as ammonium nitrate at tillering (GS23) and ammonium nitrate (AN), ammonium sulfate (AS) or urea (U) at stem elongation (GS 32), were compared with four slow release (SR) fertiliser treatments with different chemical and physical mechanisms of action: ammonium nitrate with nitrification inhibitors (SR-NI), ammonium sulphate-nitrate with nitrification inhibitors (SR-NSI), ammonium sulphate-

nitrate with double semipermeable coatings (SR-DC) and organic-mineral fertilisers (SR-OM) applied only at tillering (GS 23, 130 kg N ha^{-1}). All the previous reported treatments received the same total N rate (130 kg N ha^{-1}), according to the conventionally N rate applied in the growing areas (Blandino *et al.*., 2015).

All the top-dressed granular fertilisers were applied to experimental plots by hand. An unfertilized control (N0) that had not received any mineral N fertilisation during the growing season was introduced as a spy-control for both varieties, in order to indicate the soil fertility and N availability in the compared environments, on the basis of the canopy greenness evolution, during the grain filling stage. A second N treatment (N100) control, which had received a lower N rate (100 kg N ha^{-1}), that is, split 50 kg N ha^{-1} at GS 23 and 50 kg N ha^{-1} at GS 32 as ammonium nitrate, was also adopted (Table S1).

The N treatments were assigned to experimental units using a completely randomised block design with four replicates. The plot size was 7 x 1.5 m. The plots were seeded after an autumn ploughing (30 cm) and disk harrowing to prepare a suitable seedbed, following a previous maize crop for grain. Sowing was conducted in 12 cm wide rows (on October 27, November 3, November 6 in 2013, 2014 and 2015, respectively) at a seeding rate of 450 seeds m^{-2}. The experimental field received 35 kg ha^{-1} of P_2O_5 and 66 kg ha^{-1} K_2O each year. The weed control was conducted with tifensulfuron metile and tribenuron metile (Granstar Ultra Sx, Cheminova Agro Italia S.r.l.) at wheat tillering (GS 23). All the plots were treated, at wheat anthesis, with a fungicide mixture containing prothioconazole and tebuconazole (Prosaro®, Bayer, Italy, applied at 0.100 kg of each AI ha^{-1}) to avoid Fusarium Head Blight infection and protect against flag leaf greenness.

2.2. Weather conditions

The three growing seasons differed as far as the total rainfall that occurred in the vegetative stages (November-March) is concerned, with 524 mm in 2013/14, 725 mm in 2014/15 and 195 mm in 2015/16 (Figure 1). The rainfall distribution during the reproductive stages (April-June) was higher in 2013/14 (366 mm) than in 2014/15 (257 mm) or in 2015/16 (291 mm). The 2014/15 growing season was the one with the highest number of growing degree days (GDD), in particular in the reproductive stage (April-June).

2.3. Canopy NDVI

A hand-held optical sensing device, by GreenSeekerTM® (Trimble©, Sunnyvale, California, USA), was used to measure every 7 days the normalised difference vegetation index (NDVI) from anthesis (GS 61) to the end of the grain filling stage (the complete crop senescence, GS89). 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 (Marinaccio *et al.*, 2015). The Area Under Canopy Greenness Curve (AUCGC) during grain filling was calculated for each treatment, starting from the NDVI measurement for each observation date and using the following formula:

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

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

2.4. Grain yield

The grain yields were obtained by harvesting the whole plot with a Walter Wintersteiger cereal plot combine-harvester on July 10, June 29 and July 1 in 2014, 2015 and 2016, respectively. Grain moisture was analysed using a Dickey-John GAC2100 grain analyser (Dickey-John Corp. Auburn, IL, USA), according to the supplied programme and after a validation with reference materials. The grain yield results were adjusted to a 13% moisture content. The harvested grains were mixed thoroughly and 2 kg grain samples were taken from each plot for the qualitative analyses.

2.5. Nitrogen agronomic efficiency (NAE)

Nitrogen agronomic efficiency (NAE) was calculated as the ratio of grain yield at N treatment minus the grain yield at zero N to the quantity of the applied N for each N treatment, according to Delogu *et al.* (1998).

2.6. Small-scale quality analyses

The test weight (TW) was determined by means of a Dickey-John GAC2100 grain analyser. The thousand-kernel weight (TKW) was determined on two 100-kernel sets for each sample using an electronic balance. The number of grains per m^2 (GN) was calculated by dividing GY by grain weight. The grain protein content (GPC) was determined according to AACC 39-10 (AACC, 2000) on wholegrain flour.

Grain samples (1750 g) from each plot were milled using an experimental Bona 4RB mill (Bona, Monza, Italy), after tempering according to their hardness. The rheological properties, dough strength (W) and ratio between dough tenacity and extensibility (P/L) of the refined flours were evaluated using a Chopin alveograph, according to ICC-121 (ICC, 1992).

2.7. Analysis of the gluten proteins

Storage protein composition was determined, according to De Santis *et al.* (2018). Briefly, 100 mg of flour was suspended in 0.4 mL of KCl buffer (pH 7.8) and centrifuged to remove soluble proteins. KCl-insoluble fraction was suspended in 1-propanol solution (50% v/v) and centrifuged for 10 min at 4500 g (repeated twice) and gliadins were collected. Glutenins were extracted from the pellet by extraction solution (1-propanol 50% v/v, 1% DTT), after centrifugation at 10,000 g for 10 min (room temperature). Extracted glutenins and gliadins were quantified by Bradford method and gliadin-to-glutenin ratio (glia/glut) was determined. The glutenin subunits were separated by SDS-PAGE (T 12%, C 1.28%), at 25mA (4 hours at 10° C) using an SE 600 apparatus (Hoefer, Inc., Holliston, MA, USA). Gels were stained with Coomassie Brilliant Blue G250 and digitally acquired (Epson Perfection V750pro). HMW-GS allelic configuration of the and Glu1 Quality score was determined according to Payne and Lawrence (1983). Relative sub-unit expression was performed by densitometric analysis by software ImageQuantTL (GE Healthcare, Bio-sciences AB). Gels were subdivided into high (HMW-GS) and low (D-type, B-type, C-type) molecular weight glutenins. Glutenin composition was assessed in terms of ratios between: a) HMW-GSs Dx2 and Bx7 (H Dx/By); b) HMW-GS and LMW-GS (H/L); c) HMW-GS and the B-type LMW-GS (H/L b); d) LMW-GS B-type and C-type (L b/c). Three biological and two technical replicates were adopted.

2.8. Statistical analysis

Homogeneity was evaluated by means of Leneve's test. Analysis of the variance (ANOVA) was performed in a completely randomised block, with the genotype and N treatment as independent factors. Tukey's test was adopted as a *post hoc* test at a 0.05 level of significance. The percent changes due to the sulphur (S65, average effect of the AN-AS and SR-NSI fertilisation treatment) were assessed in relation to the S0 rate ones (AN-AN, SR-

NI), while the percent changes due to the slow release (SR treatments, as average effect of SR-NI, SR-NSI, SR-DC, SR-OM) were assessed in relation to the AN-AN standard N fertilisation; differences in percent changes were compared by Student's T-Test. The Pearson correlation analysis between the quality parameters was also performed. Statistical analysis was performed by means of JMP software (Version 8.0.2, SAS Institute Inc., 2009).

3. Results

3.1. Physiological and agronomic performance

The ANOVA generally showed a significant effect of the main factors and of their interactions on most of the investigated agronomic and grain quality parameters (Table 2). A significant impact on the agronomic and quality parameters was observed in relation to environmental variability. In 2013/14, when higher and better distributed rainfall occurred during the reproductive stage than during the other crop seasons (Fig.1), higher cumulated NDVI (AUCGC), grain yield and agronomic N use efficiency as well as lower test weight, grain protein content and HMW-GS to LMW-GSs ratios were observed (Table 3). On the other hand, a marked reduction in thousand kernel weight occurred in 2014/15, as a consequence of the higher growing degree days during the ripening stage, which led to a quick senescence of the crop. The two cultivars showed a comparable grain yield, with higher values in late maturity Sy Alteo only in 2013/14 (Table 3). Instead, differences between the two genotypes were observed in terms of thousand kernel weight, with lower values in Artico, as a consequence of the lower NDVI during ripening than in Sy Alteo (Table 3). Conversely, Artico resulted in a higher number of grains per square meter (GN) than Sy Alteo in 2014/15 and 2015/16.

N fertilisation strategies showed a significant influence on the agronomic performance of the two wheat cultivars. The highest grain yield was observed for the use of the ammonium sulphate (AN-AS) treatment. The grain yield performance of AN-AN and AN-U was not significantly different (Table 3). The effect of the application of slow release fertilisers at the tillering stage did not determine any decrease in grain yield with respect to the standard split AN-AN.

NAE was influenced above all by the Y and N factors; the GxY and YxN interactions also resulted to be significant (Table 2). The late maturity Sy Alteo cultivar showed a higher NAE in 2013/14 and in 2015/16 than cv. Artico. The highest NAE value was achieved for the AN-AS treatment (in particular in 2013/14). The use of slow release fertilizer at GS 23 showed a significant reduction in NAE compared to AN-AN, albeit only in 2015/16, which was characterised by less rainfall during the vegetative stages (Table 3). A relationship of the measured NDVI values (AUCGC) between grain yield and NAE was observed during grain filling (Fig.2), with longer stay green in Sy Alteo and for the 2013/14 growing season (Table 3). The test weight was influenced more by Y than by other effects (Table 2), and no varietal differences were observed (Fig. 3).

3.2. Grain protein content and rheological quality

Both cultivars showed low grain protein values (8.8% - 11.0%), according to the biscuit supply-chain requirements, with a marked impact of the N fertilisation (Table 2). In 2013/14 the highest grain protein content (Table 3) was observed for the S supply (AN-AS). With the exception of SR-OM in 2015-16, significantly lower values were observed in all the growing seasons in relation to all the slow release treatments, with respect to the standard AN-AN treatment (Table 3). The gluten technological characteristics were assessed in relation to the dough strength (W) and the ratio between tenacity and elasticity (P/L). W was largely influenced by the N factor; the GxY and GxN interactions were also significant (Table 2). Artico showed higher values than Sy Alteo in 2014/2015, but lower values in 2015/16 (Table 3), while a contrasting response of the two cultivars was observed for some N treatments (Fig. 3). The P/L ratio was mainly dependent on the genotype effect (Table 2), with as expected markedly higher values being observed in Sy Alteo (Table 3 and Fig. 3). Mean higher P/L values were observed in 2013/14 (Table 3). Moreover, N fertilisation showed a significant influence on the gluten properties. The S supply, in both the AN-AS and SR-NSI treatments (Table 3), showed a slight increase in W (+15%) and a marked mean reduction in P/L (-33%) (Fig. 4). In AN-AS treatment the percentage of samples with grain protein content above 10.5% and W above 110 J 10^{-4} was 75% and 54%, respectively, while AN-AN treatment exceeded these thresholds in 29% and 25% of cases, respectively. The treatment with urea showed no significant changes in W with respect to AN-AN (Fig.3). The application of slow release fertilisers showed a general reduction of -15% of W. With these application strategies, 98% of samples for grain protein content (10.5%) and 93% for W (110 J 10^{-4}) did not exceed the qualitative thresholds. Conversely, but only for Sy Alteo cv., characterized by high dough tenacity, also these fertilisation strategies had an impact on the P/L values according to the S distribution: SR-NSI (with S) significantly reduced this parameter, while SR–NI significantly increased it, with respect to the standard split N treatments (Table 3, Fig.3).

3.3. Gluten protein composition

ANOVA showed significant differences, in terms of gluten protein composition (Table 2). The gliadin-to-glutenin ratio (glia/glut) was mainly influenced by the Y and N effects and, to a lesser extent, by the GxY and YxN interactions. Higher values were generally observed for 2013/14, with no significant mean differences between the two cultivars (Table 3). The N fertilisation showed a great impact on the glia/glut ratio, with generally higher values for slow release fertilisers with respect to the standard split strategies (Fig. 4). The electrophoretic profile of HMW-GSs showed that both genotypes are characterised by the null allele at *Glu-A1*, 2+12 at *Glu-D1*; slight differences were instead observed in relation to *Glu-B1* gene for Sy Alteo characterised by the 7+9 allele and for Artico characterised by 7+8, thus a slight difference was determined in terms of Glu-1 quality score (5 for Sy Alteo, 6 for Artico). The HMW-GS expression, expressed as H/L and H/L b, was influenced to a great extent by the year (Table 2), with mean lower values in 2013/14 (Table 3). The highest H/L and H/L b were observed for Sy Alteo in 2015/16 (Table 3), and for the SR-OM treatment (Fig. 3). The S supply influenced glutenin expression to a great extent, with significantly lower H/L and H/L b ratios in the AN-AS treatment than the AN-AN one (Fig. 4). The HMW-GS composition, in terms of the Dx2-to-Bx7 ratio, and the ratio between B-type LMW-GS and C-type LMW-GS (L b/c), were mainly affected by the genotype (Table 2), with higher values in Sy Alteo for all the years (Table 3) and for all the N fertilisation treatments (Fig. 3).

Correlation matrix of the quality parameters is reported in Table 4. Dough strength (W) was highly correlated with grain protein content, but negatively correlated with P/L and glia/glut. P/L showed a highly significant positive correlation with the ratio between Dx2 and Bx7 HMW-GS (H Dx/Bx) and with the ratio between the B-type and C-type LMW-GS (L b/c).

4. Discussion

A detailed investigation on the effects of different N fertilisation strategies on grain yield, protein content and composition in relation to rheological properties of soft wheat cultivars, was carried out. The relationship between the canopy NDVI measured during the reproductive stage and grain yield confirmed the effectiveness of phenotyping for the physiological parameters of crops, not only to monitor the crop status, but also to predict the final cereal production, as has already been pointed out in literature (Marinaccio *et al*., 2015). The higher water availability in the reproductive stages observed for 2013/2014 positively influenced the canopy stay green and thus the TKW and grain yield, particularly for late maturity cultivar. Grain yield response resulted mainly related to the number of grains per square meter (Giunta et al., 2019) and this yield component, in addition to genotype and crop seasons, was more markedly influenced by N fertilization than grain weight. This response is in agreement with the results of Lopez-Bellido *et al*. (2005) and Triboi *et al*. (2006), who observed seasonal variations in the GY and yield components in relation to the rainfall distribution during vegetative and reproductive stage under different N management strategies. Within the conventional fertilizers, the use of ammonium nitrate led to a not significant variation in GY and its components with the respect to the urea, confirming, however, the trend of slight higher efficiency of the AN form (Rekowski et al., 2020); on the other hand, the application of ammonium sulphate lead to a grain yield advantage compared to ammonium nitrate in certain environmental conditions. It has been reported that a moderate increase in grain yield may be observed when N+S are co-applied, together with an increase in nitrogen use efficiency (Salvagiotti *et al*., 2009; Duncan *et al*., 2018). The same behaviour was observed with the S application within the slow-release fertilizers, in particular with the single application of SR-NSI, which showed a positive response in terms of agronomic and quality performances (Weber et al., 2008), especially with the respect to the double semi-permeable coated and the organic-mineral slow-release fertilizers, confirming the lower efficiency of N applied in organic form (Godfrey et al., 2010).

On the other hand, the present study clearly shows that the type of N fertiliser and its application strategies affect clearly the protein content and composition, even for a qualitative category with a low GPC requirement, such as the wheat used for biscuits. The observed response to different fertilisation strategies, including single N applications with the use of slow-release products and the role of S, showed a great impact on the protein composition (glutenin) and, as a consequence, on the dough properties (Xue *et al*., 2016). The gluten content and composition and the alveographic traits showed a clear interaction between fertilization, cultivar with environmental conditions. Exploring favourable interactions between genotype and management can be useful to make a contribution towards optimizing agronomic strategies for specific supply-chains, such as the biscuit making supply chain, which is characterised by low grain protein content, W and P/L requirements (Foca *et al*., 2007).

4.1. Strategies to guarantee low protein content and dough strength

As far as biscuit-making quality is concerned, Igrejas *et al*. (2002) stated that the total grain protein content is more important than the protein composition. The same authors stated that the allelic composition has little impact, in terms of dough quality. Both of the investigated cultivars, which are commercially utilised for the biscuit supply-chain, showed suitable GPC (< 10.5%) and W (< 110 J 10^{-4}) values for biscuits, resulting in 80% of the cases below optimal threshold for this category.

Varietal differences, in terms of gluten composition, resulted in differences in the alveographic parameters, although these differences also depended on the fertilisation strategies. This different behaviour is in agreement with the results of Pedersen and Jorgensen (2007), who observed differences in genotypic response to N fertilisation in terms of rheological properties in wheat cultivars for biscuits. However, the same authors did not find any significant differences in the protein content of the same cultivars, thus suggesting possible implications of the gluten composition. In our study, we have shown that the technological properties of soft wheat could be influenced both by the effect of fertilization strategies on grain protein content and on the differential expression of specific glutenin subunits. Cho *et al*. (2018) also reported changes in the proportion of high and low glutenin subunit expression due to N fertilisation and their effects on quality.

Within split fertilization strategies, the use of urea with respect to ammonium nitrate showed not significant minor changes, with a trend of reduction of GPC and alveographic strength, which are desirable for biscuit-making, and, possibly explainable by the lower efficiency of urea (Rekowski *et al*., 2020); on the contrary, in late maturity cultivar under more favourable conditions, the application of ammonium sulphate was associated to a slight higher GPC. This response, associated to an increase in glutenin content (low glia/glut), especially B-type LMW-GS, explain the slight increase in dough strength (Godfrey *et al*., 2010).

However, the effect of slow-release in comparison to conventional fertilisers, resulted in a clear reduction in GPC and W; these quantitative changes were associated with an increase in the gliadin-to-glutenin ratio, which was due to a decrease in the glutenin amount. In fact, the single N supply at an early developmental stage may have reduced glutenin accumulation, which generally occurs during late grain filling (Abonyi *et al*., 2007; Fois *et al*., 2011; Giuliani *et al*., 2015). According to our data, this management solution, with the single distribution of all N rate in an early growth stage, did not lead to any significant advantages in both grain yield or nitrogen agronomic efficiency, but it is able to guarantee the quality requirements of the wheat for biscuit wheats with greater consistency in different environmental conditions. No marked differences in rheological traits have been observed within slow release fertilizers with different chemical and physical mechanisms of action (nitrification inhibitors, double semipermeable coatings or organic-mineral fertilisers) in growing seasons with different meteorological trends, except for a trend of lower W and higher gliadin content, which can result favourable for biscuit-making quality (Labuschagne *et al.*, 1997).

4.2. Fertilization strategies to mitigate high P/L values

Low tenacity and low alveographic P/L of dough represent an important quality trait for biscuit-making. The hypothesis that biscuit-making quality is mainly determined by protein content rather than protein composition (Igrejas *et al.*, 2002) does not seem confirmed in this study. Although both investigated cultivars resulted characterized by low protein content and dough strength, only Artico satisfied the requirements for biscuit production in relation to the P/L ratio, that is, below 0.5 (Foca *et al.*, 2007). Indeed, Sy Alteo reported high P/L values (1.7 on average), strongly influenced by environmental conditions. The slight genetic differences in the *Glu-B1* HMW-GS allelic composition (7+8, 7+9) does not explain the observed differences in terms of P/L; furthermore, the same cultivars showed the null *Glu-A1* allele, which has been indicated to be suitable for biscuit-making (Zhang *et al.*, 2018). The genotype difference in P/L was mainly associated with glutenin subunit expression rather than with the allelic composition (Xue *et al.*, 2016). The different proportion of Dx2 to Bx7 HMW-GSs and of the B-type to the C-type LMW-GSs significantly affected P/L. In this study the former was found to be related more to genetic differences, while the latter environment and management. Wieser and Kieffer (2001) showed the relationship between gluten protein expression and the rheological parameters.

As for the effect of N fertilization management, results observed under the present study demonstrated how S supply can have an impact on dough quality also in wheat cultivars for biscuit supply-chain. Changes in glutenin composition due to different S supply resulted in an increased expression of sulphur-rich proteins (B- and C-type LMW-GS), with a consequent reduction in the H/L and H/L b ratios. The balance of N with S in wheat nutrition can influence the gluten protein composition and determine a reduction in the HMW-GS-to-LMW-GS ratio (Triboi *et al.*, 2000; Godfrey *et al.*, 2010; Yu et al., 2018). Within S-rich prolamins, LMW-GSs are characterised by eight cysteine residues and have a key role on determining technological performances on dough for their ability to form disulphide bonds with other polymeric proteins (D'Ovidio and Masci, 2004). In this study, the higher LMW-GS expression observed with the use AS and ASN fertilizers might have determined variation in polymerization of gluten and then an increase of extensibility (Pompa *et al.*, 2009; Zorb *et al.*, 2009). Also the regulation was influenced by S supply of specific HMW-GS subunits, such as Bx7, emerged as a key factor in influencing the alveographic performance (Li et al., 2020), and P/L in particular, which is highly relevant for technological performances in biscuit-making (Cho *et al.*, 2018). The consequent rheological changes consisted of a reduction in the dough tenacity to the extensibility ratio (P/L), even in cv. with high dough tenacity such as Sy Alteo, and in agreement with the observation of Zhao *et al.* (1999). These results help to understand the role of N and S fertilization on the influence of storage protein composition on rheological quality of minor supply chain, such as biscuit-making. This effect suggests the possibility of consider moderate sulphur co-applications to reduce P/L for specific genotypes characterized by higher dough tenacity.

Finally, the different varietal response, in relation to N fertilisation, confirms the complexity of determining wheat quality and the importance of studies that consider the effects of the interactions GxYxM (Chope *et al.*, 2015) for the different supply-chains.

5. Conclusions

The results of this study show the influence of N fertilisation, in terms of N source and application strategies, on the storage protein content and composition in relation to technological dough quality in wheat cultivars used for biscuit production. N fertilisation may be modulated to obtain high grain yields with a suitable grain protein content and composition for this specific supply chain. The observed differences in storage protein expression, especially in the glutenin subunits, showed a significant impact on dough quality, albeit more in terms of P/L than of W.

The use of slow release N fertilisers can be recommended for a suitable biscuit making quality wheat, since was particularly useful to reduce grain protein content and W, without negatively affecting the grain yield. In addition, a moderate sulphur supply may be suggested for cultivars characterised by an excessive P/L to modulate both LMW-GS expression and dough extensibility, in order to satisfy specific supply chain requirements. The marked interactions observed between fertilization management, genotype and environment suggests the need for further studies to find the best combination of agronomic practices and suitable genotypes to achieve a better stability of grain quality in wheat for biscuit production.

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References

AACC. 2000. Approved Methods of the American Association of Cereal Chemists, 10th ed., The Association: St Paul, Minnesota.

Abonyi, T., Kiraly, I., Tomoskozi, S., Baticz, O., Guoth, A., Gergely, E. S., Lasztity, D., Lasztity, R., 2007. Synthesis of gluten-forming polypeptides. 1. Biosynthesis of gliadins and glutenins subunits. J. Agric. Food Chem. 55, 3655−3660.

Barak, S., Mudgil, D., Singh, Khatkar, B., 2013. Effect of composition of gluten proteins and dough rheological properties on the cookie-making quality. British Food Journal, 115 (4), 564-574. DOI 10.1108/00070701311317847.

Blandino, M., Marinaccio, F., Vaccino, P., Reyneri, A., 2015. Nitrogen fertilization strategies suitable to achieve the quality requirements of wheat for biscuit production. Agron. J. 107, 1584-1594. DOI:10.2134/agronj14.0627.

Calabi-Floody, M., Medina, J., Rumpel, C., Condron, L.M., Hernandez, M., Dumont, M., Mora, M., 2018. Smart fertilizers as a strategy for sustainable agriculture. Adv. Agron. 147, 119-157.

Cho, S.W., Kang, C.S., Kang, T.K., Cho, K.M., Park, C.S., 2018. Influence of different nitrogen application on flour properties, gluten properties by HPLC and end-use quality of Korean wheat. Journal of Integrative Agriculture 17(5), 982–99. DOI: 10.1016/S2095-3119(18)61920-3.

Chope, G.A., Wan, Y., Penson, S.P., Bhandari, D.G., Powers, S.J., Shewry, P.R., Hawkesford, M., 2015. Effects of Genotype, Season, and Nitrogen nutrition on gene expression and protein accumulation in wheat grain. J. Agr. Food Chem. 62, 4399−4407. DOI: 10.1021/jf500625c.

Delogu, G., Cattivelli, L., Pecchioni, N., De Falcis, D., Maggiore, T., Stanca, A.M., 1998. Uptake and agronomic efficiency of nitrogen in winter barley and winter wheat. Eur. J. Agron., 9, 11–20. DOI: 10.1016/S1161-0301(98)00019-7.

De Santis, M.A., Giuliani, M.M., Giuzio, L., De Vita, P., Flagella, Z., 2018. Assessment of grain protein composition in old and modern Italian durum wheat genotypes. Italian J. Agron. 13, 308. DOI: 10.4081/ija.2018.908.

D'Ovidio, R., Masci, S., 2004. The wheat low-molecular-weight glutenin subunits. J. Cereal Sci. 39, 321–339. DOI: 10.1016/j.jcs.2003.12.002.

Duncan, E.G., O'Sullivan, C. A., Roper, M.M., Biggs, J. S., Peoples, M.B., 2018. Influence of co-application of nitrogen with phosphorus, potassium and sulphur on the apparent efficiency of nitrogen fertiliser use, grain yield and protein content of wheat: Review. Field Crops Res. 226, 56-65. DOI 10.1016/j.fcr.2018.07.010.

Ercoli, L., Lulli, L., Arduini, I., Mariotti, M., Masoni, A. 2011. Durum wheat grain yield and quality as affected by S rate under Mediterranean conditions. Europ. J. Agron. 35 (2011) 63–70. DOI:10.1016/j.eja.2011.03.007.

Farrer, D.C., Weisz, R., Heiniger, R., Murphy, J.P., White, J.G., 2006. Minimizing Protein Variability in Soft Red Winter Wheat. Agron. J. 98(4), 1137–1145.

Foca, G., Ulrici, A., Corbellini, M., Pagani, M.A., Lucisano, M., Franchini, G.C., Tassi, L., 2007. Reproducibility of the Italian ISQ method for quality classification of bread wheats: An evaluation by expert assessors. J. Sci. Food Agric. 87, 839–846. DOI: 10.1002/jsfa.2785.

Fois, S., Schlichting, L., Marchylo, B., Dexter, J., Motzo, R., Giunta, F., 2011. Environmental conditions affect semolina quality in durum wheat (*Triticum turgidum* ssp. *durum* L.) cultivars with different gluten strength and gluten protein composition. J. Sci. Food Agric. 91, 2664-73. DOI 10.1002/jsfa.4509.

Gaines, C.S., 1991. Associations among quality attributes of red and white soft wheat cultivars across locations and crop years. Cereal Chem. 68, 56–59.

Garrido-Lestache, E., López-Bellido, R.J., López-Bellido. L., 2004. Effect of N rate, timing and splitting and N type on bread-making quality in hard red spring wheat under rainfed Mediterranean conditions. Field Crop. Res. 85, 213–236.

Giuliani, M.M., Giuzio, L., De Caro, A., Flagella, Z., 2011. Relationships between nitrogen utilization and grain technological quality in durum wheat: II. Grain yield and quality. Agron. J. 103, 1668–1675. DOI: 10.2134/agronj2011.0154.

Giuliani, M.M., Palermo, C., De Santis, M.A., Mentana, A., Pompa, M., Giuzio, L., Masci, S., Centonze, D., Flagella, Z., 2015. Differential expression of durum wheat gluten proteome under water stress during grain filling. J. Agric. Food Chem. 63, 6501–6512. DOI: 10.1021/acs.jafc.5b01635.

Giunta, F., Pruneddu, G., Zuddas, M., Motzo, R. 2019. Bread and durum wheat: Intra- and inter-specific variation in grain yield and protein concentration of modern Italian cultivars. Euro J. Agron.105: 119–128. DOI: 10.1016/j.eja.2019.02.011.

Godfrey, D., Hawkesford, M.J., Powers, S.J., Millar, S., Shewry, P.R., 2010. Effects of crop nutrition on wheat grain composition and end use quality. J. Agric. Food. Chem. 58, 3012–3021. DOI**:** 10.1021/jf9040645.

Hawkesford, M., 2014. Reducing the reliance on nitrogen fertilizer for wheat production. J. Cereal Sci. 59: 276-283. DOI 10.1016/j.jcs.2013.12.001.

Hurkman, W.J., Tanaka, C.K., Vensel, W.H., Thilmony, R., Altenbach, S.B., 2013. Comparative proteomic analysis of the effect of temperature and fertilizer on gliadin and glutenin accumulation in the developing endosperm and flour from *Triticum aestivum* L. cv. Butte6. Proteome Sci. 11: 8. DOI: 10.1186/1477-5956-11-8.

ICC. 1992. Standard methods of the International Association for Cereal Chemistry. The Association: Vienna.

Igrejas, G., Guedes-Pinto, H., Carnide, V., Clement, J., Branlard, G., 2002. Genetical, Biochemical and Technological Parameters Associated with Biscuit Quality. II. Prediction Using Storage Proteins and Quality Characteristics in a Soft Wheat Population. J. Cereal Sci. 36, 187–197. DOI: 10.1006/jcrs.2002.0452.

Labuschagne, M.T., Claassen, A., van Deventer, C.S., 1997. Biscuit-making quality of backcross derivatives of wheat differing in kernel hardness. Euphytica 96, 263–266. DOI: 10.1023/A:1003029618430.

Li, S., Liu, Y., Tong, J., Yu, L., Ding, M., Zhang, Z., Rehman, A., Majzoobi, M., Wang, Z., Gao, X., 2020. The overexpression of high-molecular-weight glutenin subunit Bx7 improves the dough rheological properties by altering secondary and micro-structures of wheat gluten. Food Res. Int. 130, 108914. DOI: 10.1016/j.foodres.2019.108914.

Lopez-Bellido, L., Lopez-Bellido, R.J., Redondo, R., 2005. Nitrogen efficiency in wheat under rainfed Mediterranean conditions as affected by split nitrogen application. Field Crop. Res. 94(1), 86-97. DOI: 10.1016/j.fcr.2004.11.004.

MacRitchie, F., 2016. Seventy years of research into breadmaking quality. J. Cereal Sci. 70, 123-131. DOI: 10.1016/j.jcs.2016.05.020.

Marinaccio, F., Reyneri, A., Blandino, M., 2015. Enhancing grain yield and quality of winter barley through agronomic strategies to prolong canopy greenness. Field Crops Res. 170, 109-118. DOI 10.1016/j.fcr.2014.10.002.

Payne, P.I., Lawrence, G.J., 1983. Catalogue of alleles for the complex gene loci, GLU-A1, GLU-B1, and GLU-D1 which code for high-molecular weight subunits of glutenin in hexaploid wheat. Cereal Res Commun. 11, 29–35.

Pedersen, L., Jorgensen, J.R., 2007. Variation in rheological properties of gluten from three biscuit wheat cultivars in relation to nitrogen fertilisation. J. Cereal Sci. 46, 132–138. DOI: 10.1016/j.jcs.2007.01.001.

Pompa, M., Giuliani, M.M., Giuzio, L., Gagliardi, A., Di Fonzo, N., Flagella, Z., 2009. Effect of sulphur fertilization on grain quality and protein composition of durum wheat (*Triticum durum* Desf.). Ital. J. Agron., 4, 159-170. DOI: 10.4081/ija.2009.4.159.

Rekowski, A., Wimmer, M., Hitzmann, B., Hermannseder, B., Hahn, H., Zorb, C., 2020. Application of urease inhibitor improves protein composition and breadbaking quality of urea fertilized winter wheat. J. Plant Nutr. Soil Sci., 000, 1–11. DOI: 10.1002/jpln.201900529.

Salvagiotti, F., Castellarin, J.M., Miralles, D.J., Pedrol, H.M., 2009. Sulfur fertilization improves nitrogen use efficiency in wheat by increasing nitrogen uptake. Field Crops Res. 113, 170–177. DOI: 10.1016/j.fcr.2009.05.003.

Tao, Z., Chang, X., Wang, D., Wang, Y., Ma, S., Yang, Y., Zhao, G., 2018. Effects of sulfur fertilization and short-term high temperature on wheat grain production and wheat flour proteins. The Crop Journal 6: 413–425. DOI: 10.1016/j.cj.2018.01.007.

Triboi, E., Abad, A., Michelena, A., Lloveras, J., Ollier, J.L., Daniel, C., 2000. Environmental effects on the quality of two wheat genotypes: 1. Quantitative and qualitative variation of storage proteins. Eur. J. Agron. 13, 47–64.

Triboi, E., Martre, P., Girousse, C., Ravel, C., Triboi-Blondel, A.M., 2006. Unravelling environmental and genetic relationships between grain yield and nitrogen concentration for wheat. Eur. J. Agron. 25, 108-118. DOI: 10.1016/j.eja.2006.04.004.

Weber, E.A., Koller, W., Graeff, S., Hermann, W., Merkt, N., Claupein, W. 2008. Impact of different nitrogen fertilizers and an additional sulfur supply on grain yield, quality, and the potential of acrylamide formation in winter wheat. J. Plant Nutr. Soil Sci., 171, 643–655. DOI: 10.1002/jpln.200700229

Wieser, H., Kieffer, R., 2001. Correlations of the amount of gluten protein types to the technological properties of wheat flours determined on a micro-scale. J. Cereal Sci. 34, 19–27. DOI: 10.1006/jcrs.2000.0385

Wieser, H., 2007. Chemistry of gluten proteins. Food Microbiology, 24, 115-119. DOI 10.1016/j.fm.2006.07.004.

Xue, C., Schulte auf'm Erley, G., Rossmann, A., Schuster, R., Koehler, P., Muehling, K.H., 2016. Split nitrogen application improves wheat baking quality by influencing protein composition rather than concentration. Front Plant Sci. 738 (7), 1-11. DOI: 10.3389/fpls.2016.00738

Yu, Z., Juhasz, A., Islam, S., Diepeveen, D., Zhang, J., Wang, P., Ma, W., 2018. Impact of mid-season sulphur deficiency on wheat nitrogen metabolism and biosynthesis of grain protein. Sci. Rep. 8, 2499. DOI:10.1038/s41598-018-20935-8

Zadoks, J.C., Chang, T.T., Konzak, C.F., 1974. A decimal code for the growth stages of cereals. Weed Res. 14, 415–421.

Zhang, X., Zhang, B., Wu, H., Lu, C., Liu, G., Li, M., Jiang, W., Song, G., Gao, D., 2018. Effect of high-molecular-weight glutenin subunit deletion on soft wheat quality properties and sugar-snap cookie quality estimated through near-isogenic lines. Journal of Integrative Agriculture, 17(5), 1066–1073. DOI 10.1016/S2095-3119(17)61729-5.

Zhao, F.J., Salmon, S.E., Withers, P.J., Evans, E.E., Monaghan, J.M., Shewry, P.R., McGrath, S.P., 1999. Responses of breadmaking quality to sulphur in three wheat varieties. J. Sci. Food Agric. 79, 1865-1874.

Zorb, C., Steinfurth, D., Seling, S., Langenkamper, G., Koehler, P., Wieser, H., Lindhauer, M.G., Muhling K.H., 2009. Quantitative protein composition and baking quality winter wheat as affected by late sulphur fertilization. J. Agric. Food Chem. 57, 3877-3885. DOI:10.1021/jf8038988.

Zorb, C., Ludewig, U., Hawkesford, M., 2018. Perspective on Wheat Yield and Quality with Reduced Nitrogen Supply. Trends Plant Sci. 11 (23), 1029-1037. DOI: 10.1016/j.tplants.2018.08.012.

List of figures.

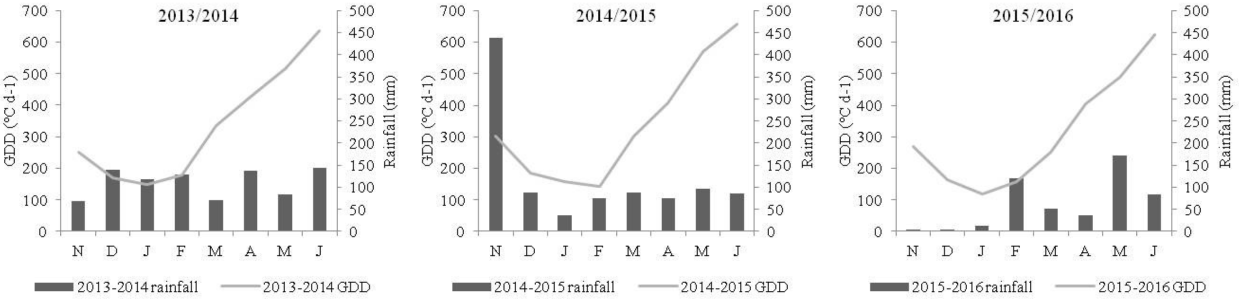

Fig. 1. Monthly rainfall distribution and growing degree days (GDD[a]) from each crop seasons.

[a] Accumulated growing degree days for each month using a 0°C base.

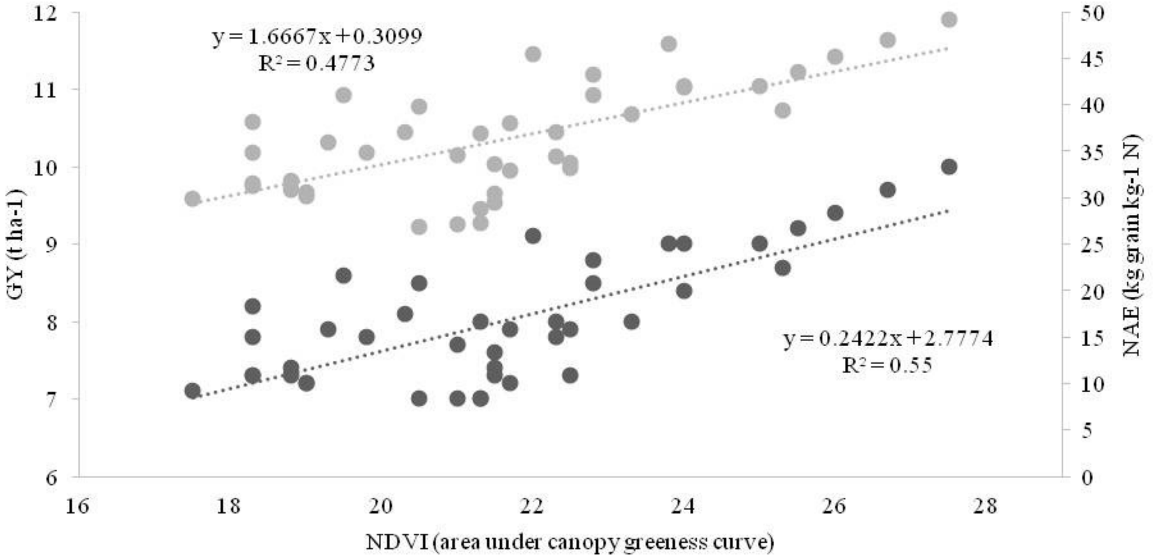

Fig. 2. Relationship between the area under canopy greenness curve (AUCGC) obtained from NDVI measurements from anthesis to the complete crop senescence, with grain yield (GY, black) and nitrogen agronomic efficiency (NAE, grey) of two soft wheat genotypes under N fertilization strategies.

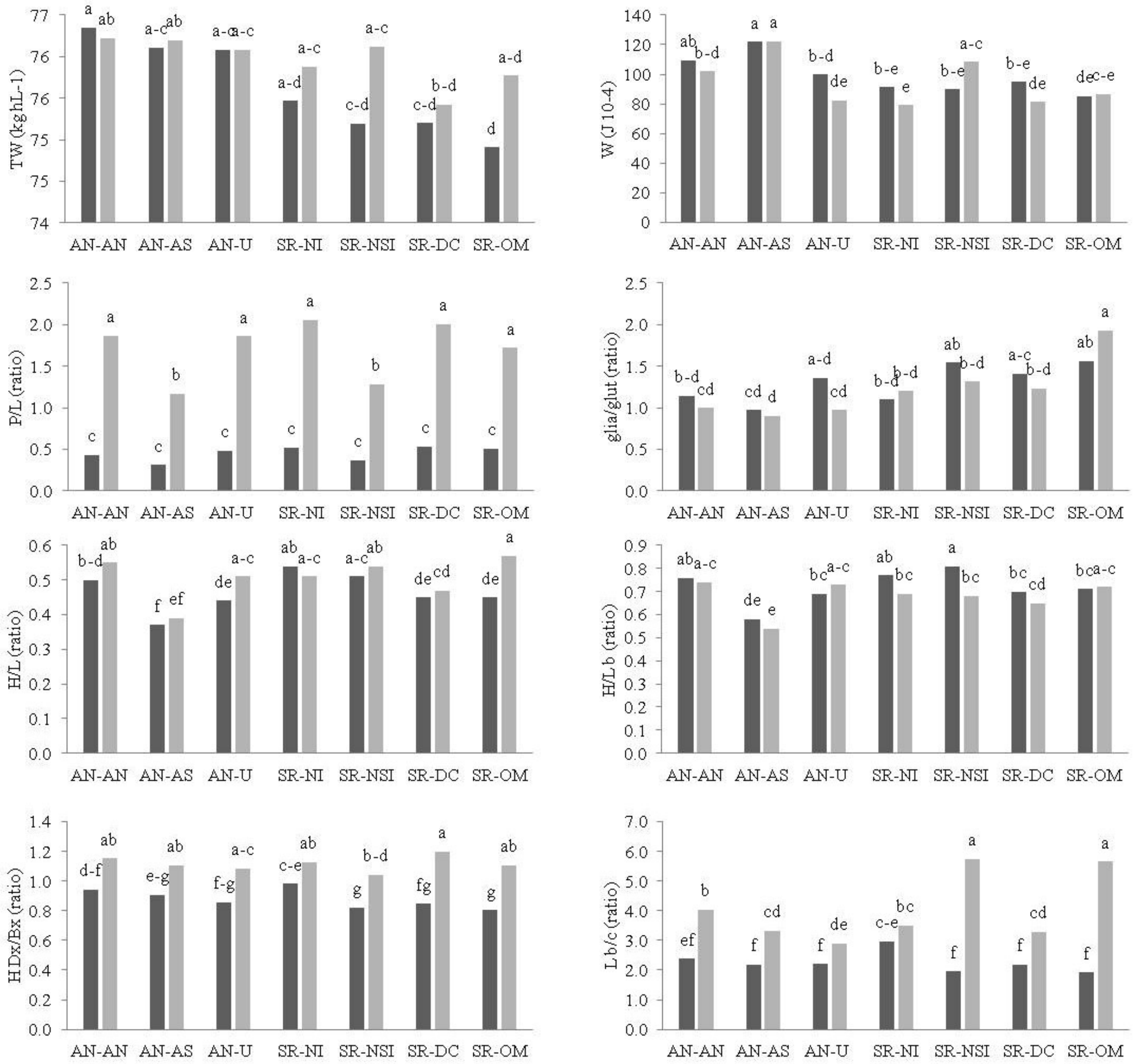

Fig. 3. Effect of the genotype x N fertilization (GxN) interaction on test weight (TW), alveographic parameters (W, P/L) and gluten composition of the two wheat cultivars under study (Sy Alteo and Artico).

See table 1 for the details of compared N fertilization strategies. W = dough strength; P/L = ratio between dough tenacity and extensibility; glia/glut = gliadin to glutenin ratio; H/L = ratio between HMW-GS to LMW-GS; H/L b = ratio between HMW-GS to B-type LMW-GS; H Dx/Bx = the ratio between Glu-Dx2 and Glu-Bx7 HMW-GSs; L b/c = ratio between B-type and C-type LMW-GSs.

Levels not connected by the same letters are significant different according to Tukey's test at 5% level of significance. When present, the dash replaces the intermediate letters in alphabetical order among those reported.

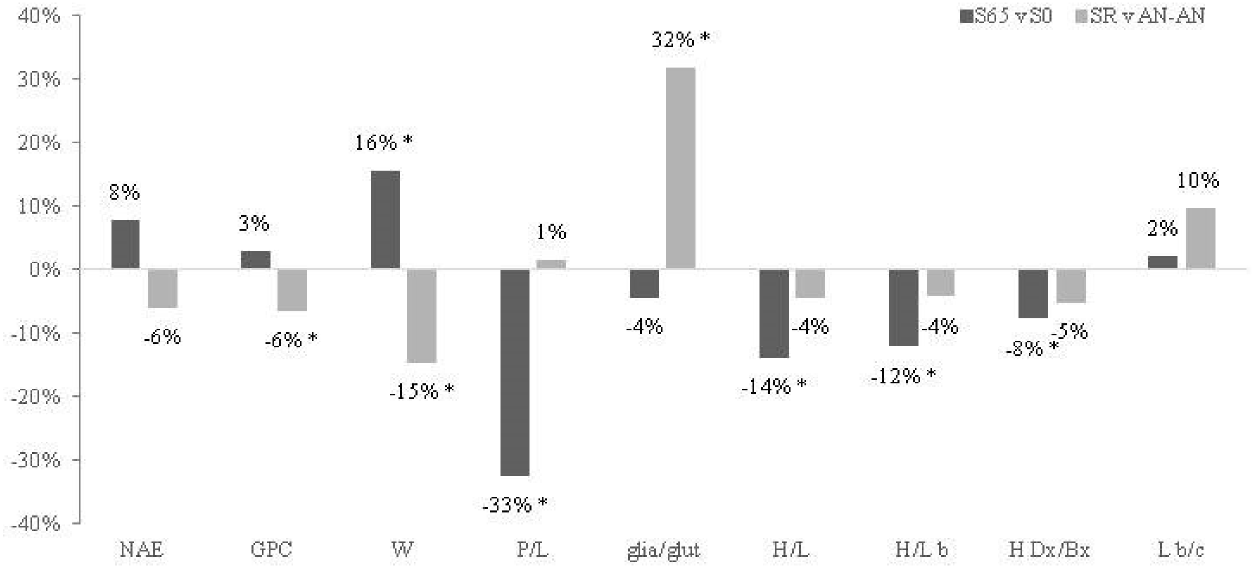

Fig. 4. Percent change due to the effect of sulphur (S) application at stem elongation stage (S65 vs S0) and to the slow release N fertilization strategies (SR) compared to split ammonium nitrate application (SR vs AN-AN) on the main agronomic and quality parameters of soft wheat under study

Mean changes for S effect were calculated by comparing means of S65 rate treatments (AN–AS, SR-NIS) with S0 rate ones (AN-AN, SR-NI), while mean changes for SR effect were calculated by comparing mean of SR treatments (SR-NI, SR-NIS, SR-DC, SR-OM) with the standard AN-AN treatment.

NAE = nitrogen agronomic efficiency; GPC = grain protein content; W = dough strength; P/L = ratio between dough tenacity and extensibility; glia/glut = gliadin to glutenin ratio; H/L = ratio between HMW-GS to LMW-GS; H/L b = ratio between HMW-GS to B-type LMW-GS; H Dx/Bx = the ratio between Glu-Dx2 and Glu-Bx7 HMW-GSs; L b/c = ratio between B-type and C-type LMW-GSs.

* = differences significant at 0.05 according to Student’s T-Test .

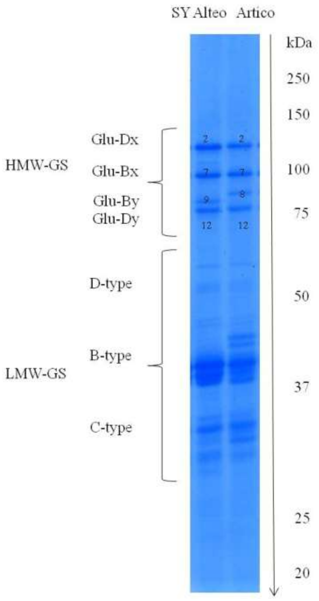

Fig. S1. Electrophoretic separation by SDS-PAGE of soluble glutenin from two investigated soft wheat cultivars (Sy Alteo, Artico) and their relative allelic HMW-GS configuration.

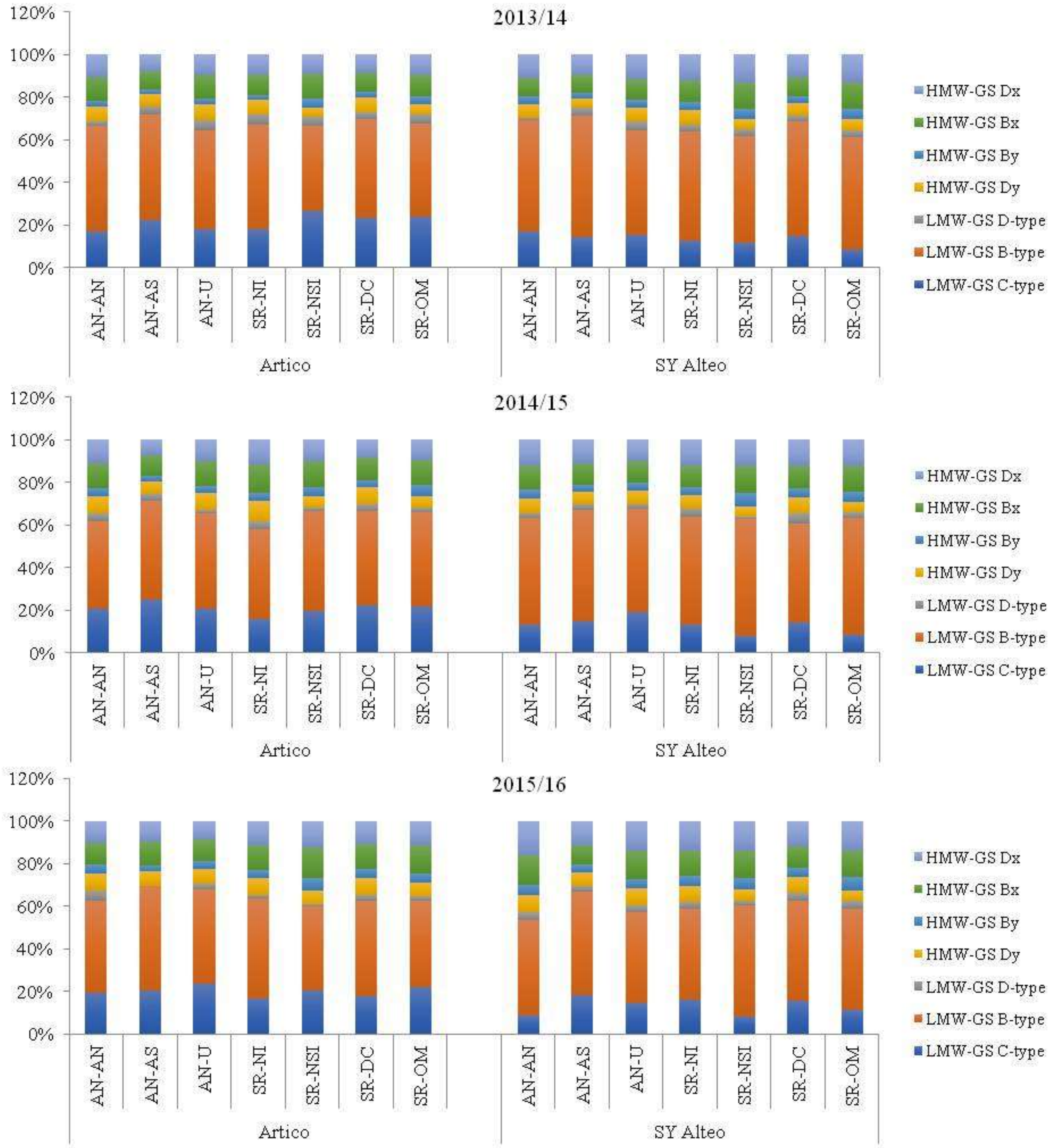
2013/14
2014/15
2015/16
120%
100%
80%
60%
40%
20%
0%
AN-AN
AN-AS
AN-U
SR-NI
SR-NSI
SR-DC
SR-OM
Artico
SY Alteo
HMW-GS Dx
HMW-GS Bx
HMW-GS By
HMW-GS Dy
LMW-GS D-type
LMW-GS B-type
LMW-GS C-type

Fig. S2. Mean of glutenin subunits expression of soft wheat genotypes (Artico, SY Alteo) under different N fertilization strategies in three crop seasons (2013/14, 2014/15, 2015/16).

In order from the top to the bottom: HMW-GS Dx2; HMW-GS Bx7; HMW-GS By (8 for Artico, 9 for SY Alteo); HMW-GS Dy12; LMW-GS D-type; LMW-GS B-type; LMW-GS

List of tables.

Table 1.

List of N fertilisation strategies adopted in the field trial of the two soft wheat cultivars under study.

N treatment	N source		N rate kg ha^{-1}			S rate kg ha^{-1}		
	tillering (GS23)	stem elongation (GS32)	GS23	GS32	total	GS23	GS32	total
AN-AN	ammonium nitrate [a]	ammonium nitrate [a]	50	80	130	0	0	0
AN-AS	ammonium nitrate	ammonium sulphate [b]	50	80	130	0	65	65
AN-U	ammonium nitrate	urea [c]	50	80	130	0	0	0
SR-NI	ammonium nitrate + nitrification inhibitor [d]	-	130	0	130	0	0	0
SR-NSI	ammonium sulphate nitrate + nitrification inhibitor [e]	-	130	0	130	65	0	65
SR-DC	slow release fertilizer trough double membrane [f]	-	130	0	130	22	0	22
SR-OM	slow release fertilizer trough organ-mineral [g]	-	130	0	130	26	0	26

[a] using granular ammonium nitrate (AN, 26% N) top dressed at tillering and stem elongation growth stages

[b] using granular ammonium nitrate (AN, 26% N) top-dressed at tillering and a mixture of ammonium sulphate (AS; 21% N, 24% S) + ammonium nitrate top-dressed at the stem elongation

[c] using granular ammonium nitrate (AN, 26% N) top-dressed at tillering and urea (U; 46% N) top-dressed at the stem elongation stage

[d] using granular ammonium nitrate with nitrification inhibitor dicyandiamide (DCD) (Supertet 26®, Panfertil Spa, Ravenna, Italy; 26% N: 13% NO3-, 13% NH4+) top-dressed at tillering growth stage

[e] using granular ammonium sulphate-nitrate with nitrification inhibitor 3,4-dimethyilpyirazole phosphate (DMPP) (Entec 26®, EuroChem Agro Spa, Cesano Maderno, Italy; 26% N: 7.5% NO3-, 18.5% NH4+ and 13% S) top-dressed at tillering growth stage

[f] using granular urea ammonium sulphate with double organic and inorganic membrane (MeTA) (Rhizovit 35 N-process®, Timac Agro S.p.A, Atessa, Italy);

[g] using granular organic - mineral fertilizer with humified peat (Azotop 30®, SCAM Spa, Modena, Italy; 30% N: 6% NH4+, 23% ureic N, 1% organic N and 6% S, organic C 7.5%) top-dressed at tillering growth stage

Table 2. Analysis of variance (F-Statistics and p-value) relative to the genotype (G), year (Y), N fertilization treatments (N) and of their interactions.

Factors	DF	NDVI	GY	NAE	GN	TKW	TW	GPC	W	P/L	glia/glut	H/L	H/L b	H Dx/Bx	L b/c
G	1	253.3 **	19.8 **	4.1 ns	83.5 **	1820.2 **	10.8 *	2.1 ns	5.0 *	1067.8 **	3.9 ns	39.5 **	13.6 *	380.5 **	795.3 **
Y	2	206.7 **	45.5 **	53.1 **	112.4 **	928.8 **	104.9 **	75.3 **	15.7 **	63.3 **	16.2 **	109.9 **	104.1 **	30.7 *	4.0 *
N	6	7.2 **	7.9 **	7.9 **	7.4 **	1.7 ns	8.3 **	55.5 **	21.8 **	15.9 **	11.1 **	38.7 **	28.2 **	8.6 *	37.1 **
GxY	2	0.3 ns	16.6 **	7.7 *	12.8 **	32.9 **	29.3 **	25.8 **	43.3 **	50.9 **	4.1 *	41.4 **	43.8 **	2.7 ns	8.1 *
GxN	6	0.1 ns	0.4 ns	0.4 ns	0.2 ns	1.3 ns	2.3 *	1.1 ns	4.1 *	5.8 **	2.1 ns	7.9 **	5.5 **	4.5 *	66.3 **
YxN	12	3.2 *	2.4 *	2.4 **	3.1 *	2.5 *	2.9 **	7.7 **	2.9 *	1.3 ns	3.3 *	19.0 **	21.1 **	10.3 **	14.0 **
GxYxN	12	0.3 ns	0.5 ns	0.5 ns	0.4 ns	0.5 ns	1.4 ns	0.5 ns	1.2 ns	0.7 ns	1.6 ns	14.9 **	12.1 **	1.9 ns	10.0 **

DF = degree of freedom; AUCGC = area under canopy greenness curve; GY = grain yield; NAE = nitrogen agronomic efficiency; GN = grain number; TKW = thousand kernel weight; GPC = grain protein content; W = dough strength; P/L = ratio between dough tenacity and extensibility; glia/glut = gliadin to glutenin ratio; H/L = ratio between HMW-GS to LMW-GS; H/L b = ratio between HMW-GS to B-type LMW-GS; H Dx/Bx = the ratio between Glu-Dx2 and Glu-Bx7 HMW-GSs; L b/c = ratio between B-type and C-type LMW-GSs.

Level of significance: * = $P \leq 0.05$; ** = $P \leq 0.001$; ns = not significant.

Table 3. Effects of the genotype x year (GxY) and of the year x N fertilization (YxN) interactions on the main agronomic and quality parameters of the two wheat cultivars under study.

Factors	Year	Level	AUCGC	GY	NAE	GN	TKW	TW	GPC	W	P/L	glia/glut	H/L	H/L b	H Dx/Bx	L b/c
			-	t ha^{-1}	kg grain kg^{-1} N	10^{3} m^{-2}	g	kg hl^{-1}	%	J 10^{-4}	ratio	ratio	ratio	ratio	ratio	ratio
G	2013/14	Artico	22.7 b	7.9 b	38.2 b	16.5 c	48.0 c	75.1 c	9.7 c	99.3 bc	0.48 c	1.52 a	0.43 c	0.66 c	0.93 c	2.36 c
		Sy Alteo	25.7 a	9.3 a	44.0 a	16.0 c	58.0 a	74.3 d	9.3 d	88.2 cd	2.32 a	1.40 ab	0.43 c	0.58 d	1.14 a	4.01 b
	2014/15	Artico	19.1 d	8.0 b	36.3 b	21.1 a	38.1 e	75.5 c	10.3 a	103.5 b	0.53 c	1.31 b	0.48 b	0.74 b	0.79 d	2.17 c
		Sy Alteo	21.8 c	8.1 b	37.7 b	17.9 b	45.3 d	76.6 ab	10.0 b	77.6 d	1.43 b	1.20 ab	0.48 b	0.63 cd	1.07 b	4.34 a
	2015/16	Artico	18.8 d	7.4 c	29.9 c	16.6 c	44.6 d	76.2 b	9.9 b	93.6 bc	0.33 c	1.18 bc	0.49 b	0.74 b	0.91 c	2.23 c
		Sy Alteo	21.5 c	7.4 c	32.0 c	13.1 d	56.0 b	76.9 a	10.3 a	116.0 a	1.44 b	0.96 c	0.61 a	0.83 a	1.14 a	3.84 b
N	2013/14	AN-AN	24.0 a-b	8.3 b-f	38.3 b-f	15.6 d-f	52.9 a-c	75.0 f	9.8 e-j	104.0 be	1.38 a-e	1.32 b-d	0.38 ij	0.54 h-i	1.18 ab	2.93 ef
		AN-AS	25.6 a	9.5 a	47.9 a	17.9 c-e	53.3 ab	75.6 c-e	11.0 a	135.3 a	0.88 f-h	0.85 e	0.35 j	0.49 i	0.93 e-g	3.08 d-f
		AN-U	22.8 b-e	8.1 b-g	37.5 b-h	15.4 d-f	52.8 a-d	74.8 ef	9.4 ik	87.5 c-f	1.44 a-d	1.29 b-d	0.50 d-g	0.75 b-e	1.01 c-f	2.85 ef
		SR-NI	24.7 ab	8.6 a-d	41.0 abc	16.0 c-f	53.7 a	74.7 ef	9.3 jk	81.5 d-f	1.62 ab	1.39 b-d	0.43 g-j	0.56 hi	1.23 a	3.81 b-d
		SR-NSI	25.2 a	9.1 ab	44.9 ab	17.6 c-e	52.0 a-e	74.7 ef	9.3 jk	94.6 b-f	1.05 b-f	1.66 a-c	0.51 c-f	0.76 b-d	0.89 e-j	2.92 ef
		SR-DC	23.7 a-c	8.4 b-e	39.5 b-e	15.7 d-f	53.5 a	74.1 f	8.9 k	78.8 ef	1.67 a	1.29 b-d	0.37 j	0.54 hi	0.99 c-f	2.80 ef
		SR-OM	23.5 a-d	8.3 b-e	38.7 b-g	15.8 d-f	52.7 a-d	74.2 f	8.8 k	75.1 f	1.50 a-c	2.18 a	0.50 d-g	0.70 d-g	1.02 c-f	3.92 a-c
	2014/15	AN-AN	19.8 fg	8.1 b-g	37.5 c-e	19.2 a-c	43.1 f	77.1 a	10.7 a-c	100.5 b-f	1.03 d-h	0.96 cd	0.53 c-f	0.78 b-d	0.88 fg	2.91 ef
		AN-AS	20.8 e-g	8.9 a-c	43.2 a-c	21.6 a	41.8 fg	76.2 a-d	10.9 ab	116.2 ab	0.70 g-k	0.99 cd	0.39 h-j	0.58 hi	0.98 c-g	2.68 ef
		AN-U	19.6 fg	7.8 b-g	34.7 c-h	18.5 a-d	42.6 fg	76.8 ab	10.4 b-e	87.0 c-f	1.14 c-g	1.02 cd	0.40 h-j	0.61 f-i	0.90 e-g	2.40 f
		SR-NI	21.6 c-f	8.6 bc	41.6 a-c	21.7 a	41.0 fg	75.9 d-e	10.1 d-h	81.8 d-f	1.21 b-f	1.11 b-d	0.61 ab	0.85 ab	0.95 d-g	3.19 c-e
		SR-NSI	21.5 d-f	8.3 b-e	39.0 b-d	21.0 ab	40.0 g	75.7 b-e	10.0 d-h	86.3 c-f	0.63 h	1.51 b-d	0.49 e-g	0.64 e-h	0.92 e-g	4.61 a
		SR-DC	19.8 fg	7.1 e-g	30.0 d-h	16.9 c-f	41.8 fg	75.7 b-e	9.6 g-j	78.8 ef	1.16 c-g	1.44 b-d	0.47 e-h	0.72 c-f	1.04 b-e	2.62 ef
		SR-OM	20.1 fg	7.5 c-g	32.7 c-h	18.1 b-e	41.4 fg	75.2 d-f	9.6 h-j	83.3 d-f	1.00 d-h	1.78 ab	0.46 f-i	0.62 f-h	0.84 g	4.38 ab
	2015/16	AN-AN	20.8 e-g	7.9 b-g	35.2 c-h	16.3 c-f	49.5 e	76.8 ab	10.3 b-f	109.3 bc	0.98 e-h	0.93 cd	0.68 a	0.93 a	1.08 a-d	3.77 b-d
		AN-AS	21.1 ef	7.8 b-g	34.3 c-h	15.6 d-f	50.8 b-e	76.6 a-c	10.6 a-d	114.8 ab	0.64 h	0.97 cd	0.41 h-j	0.60 g-i	1.11 a-c	2.51 e-f
		AN-U	20.1 fg	7.3 d-g	30.6 d-h	15.0 ef	49.5 e	76.6 a-c	10.2 c-g	98.7 b-f	0.93 e-h	1.19 b-d	0.52 c-f	0.78 b-d	1.00 c-f	2.39 f
		SR-NI	19.0 g	7.0 g	28.3 h	14.2 f	50.3 de	76.5 a-c	9.8 f-j	92.2 b-f	1.04 d-h	0.95 cd	0.55 b-e	0.78 b-d	0.98 c-g	2.70 ef
		SR-NSI	20.3 fg	7.2 e-g	29.8 e-h	14.4 f	50.6 c-e	76.5 a-c	10.0 e-i	114.3 ab	0.76 f-h	1.13 cd	0.58 bc	0.84 abc	0.97 c-g	4.04 ab
		SR-DC	19.8 fg	7.2 e-g	29.4 gh	14.3 f	51.0 a-e	76.1 a-c	10.0 e-h	106.3 b-d	0.99 d-h	1.23 b-d	0.53 b-f	0.76 b-d	1.03 b-e	2.80 ef
		SR-OM	20.0 fg	7.1 fg	28.8 h	14.3 f	50.4 e	76.6 a-c	10.1 d-h	98.0 b-f	0.88 f-h	1.06 cd	0.57 b-d	0.82 a-c	1.00 c-f	3.06 d-f

AUCGC = area under canopy greenness curve, recorded from anthesis to complete senescence; GY = grain yield; NAE = nitrogen agronomic efficiency; GN = number of grain per square meter; TKW = thousand kernel weight; GPC = grain protein content; W = dough strength; P/L = ratio between dough tenacity and extensibility; glia/glut = gliadin to glutenin ratio; H/L = ratio between HMW-GS to LMW-GS; H/L b = ratio between HMW-GS to B-type LMW-GS; H Dx/Bx = the ratio between Glu-Dx2 and Glu-Bx7 HMW-GSs; L b/c = ratio between B-type and C-type LMW-GSs. See table 1 for the details of compared N fertilization strategies.

Levels not connected by the same letters are significant different at P ≤ 0.05 according to Tukey's test. When present, the dash replaces the intermediate letters in alphabetical order among those reported.

Table 4.

Matrix of correlation between agronomic and quality parameters of wheat cultivars for biscuit end-use, under different nitrogen fertilization strategies.

	W	P/L	glia/glut	H/L	H/L b	H Dx/Bx	L b/c
GPC	0.64 ***	-0.30 *	-0.61 **	0.06	0.08	-0.07	-0.12
W		-0.34 *	-0.49 **	0.09	0.13	-0.10	-0.05
P/L			0.01	0.09	-0.15	0.66 ***	0.50 **
glia/glut				-0.07	-0.08	-0.08	0.11
H/L					0.91 ***	0.06	0.31 *
H/L b						-0.19	-0.07
H Dx/Bx							0.46 **

Data reported in table are Pearson product-moment correlation coefficient. Data reported are based on 3 years, 2 cultivars, 7 fertilization strategies and 4 replications. GPC = grain protein content; W = dough strength; P/L = ratio between dough tenacity and extensibility; glia/glut = gliadin to glutenin ratio; H/L = ratio between HMW-GS to LMW-GS; H/L b = ratio between HMW-GS to B-type LMW-GS; H Dx/Bx = the ratio between Glu-Dx2 and Glu-Bx7 HMW-GSs; L b/c = ratio between B-type and C-type LMW-GSs. Level of significance: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

Supplementary material.

Table S1.

Mean of NDVI evolution during ripening (AUCGC), grain yield (GY) and nitrogen agronomic efficiency (NAE) in unfertilized (N0) and control (N100) treatments.

Genotype	Year	N source		N rate		AUCGC	GY	NAE
			GS23	GS32	total	-	t ha^{-1}	kg grain kg^{-1} N
Sy Alteo	2013/14	-	0	0	0	13.9	3.6	-
		AN	50	50	100	21.6	7.9	43.0
	2014/15	-	0	0	0	16.0	3.2	-
		AN	50	50	100	21.2	7.6	44.0
	2015/16	-	0	0	0	11.8	3.5	-
		AN	50	50	100	21.3	7.8	43.0
Artico	2013/14	-	0	0	0	12.2	3.0	-
		AN	50	50	100	21.1	7.7	47.0
	2014/15	-	0	0	0	11.8	3.3	-
		AN	50	50	100	21.5	7.9	46.0
	2015/16	-	0	0	0	9.7	3.2	-
		AN	50	50	100	21.4	7.8	46.0

AUCGC = area under canopy greenness curve; GY = grain yield; NAE = nitrogen agronomic efficiency, AN = ammonium nitrate (N 26%).