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Durum wheat cultivars grown in Mediterranean environments can combine high grain nitrogen content with high grain yield

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Abstract

Grain protein percentage is one of the main determinants of durum wheat grain quality. However, high grain protein percentages are generally associated with low grain yields. Our aim was to identify the genotypic constraints to the realization of high grain yields with high grain N contents. To investigate this relationship, four cultivars specifically selected for their large range in 'grain yield-grain protein' relationship were compared across three seasons in a Mediterranean environment at three nitrogen (N) fertilization rates (0, 80 and 160 kg N ha^{-1}). The genotypic superiority in protein percentage was consistently associated with lower number of grains m^{-2} and high grain weight across years, N treatments and yield levels (from 3.5 to 8.5 t ha^{-1}), whereas grain yield ranking varied with year and N treatment. A high and consistent grain protein percentage was the consequence of a high N uptake by anthesis (250 kg N ha^{-1} at the higher N rate) and was consistently associated to a low grain yield. The good capacity to absorb N after anthesis, on the contrary, resulted in a grain N percentage less reliable than that obtained through a high pre-anthesis N uptake, but still high and associated with the ability to make the most of favourable weather conditions by combining a high grain N with a grain yield comparable to those of more productive cultivars. Post-anthesis N uptake and high potential grain weight are two interesting targets in breeding for high yielding, high protein cultivars.

Keywords: grain nitrogen; NNI; grain number; nitrogen source; source:sink

1. Introduction

Grain protein percentage is one of the main determinants of durum wheat grain quality, but grain yield and grain nitrogen/protein content are often negatively correlated (Rharrabti et al., 2001; Giunta et al., 2019) as they are in other cereal crops (Triboi et al., 2006; Simmonds, 1996; Triboi & Triboi-Blondel, 2002; Guarda et al., 2004; Cooper et al., 2001; Laidig et al., 2017). This trade-off between grain yield and grain nitrogen has largely been studied in relation to breeding by comparing old and modern cultivars (Giunta et al., 2007; De Vita et al., 2007). Thus, wheat breeding that has improved yields through increases in grain number per m^2 (GNO) has not resulted in a proportional increase in total N uptake ('N source'; Sadras et al., 2016), and the consequent decrease in post-anthesis Nsource-Nsink balance has reduced the grain N content by diluting a rather limited source of nitrogen across more grains (Acreche and Slafer, 2009).

Multi-environmental varietal trials in which modern durum wheat cultivars are subjected to the same agronomic technique clearly show the consistency of the negative association between grain yield and grain N under varying environmental conditions. Some cultivars consistently produce grains with a high grain protein percentage but low to medium grain yields, whereas others are able to produce high or very high grain yields, but low grain protein percentage (Giunta et al, 2019). One weakness of this type of comparison could be the N rate adopted, because high-yielding cultivars need a larger N availability to avoid a decrease in grain protein percentage while sustaining their high grain yields (Silvester-Bradley et al., 2009).

Studies have shown that deviations from this negative relationship have a genetic basis, but their physiological basis is still poorly understood (Oury and Godin, 2007; Bogart et al., 2010).

Grain growth is sink-limited for C accumulation (Reynolds et al., 2005), whereas grain N content of modern wheat cultivars is generally considered to be 'source limited' (Martre et al., 2006). The N accumulated and stored in the plant's vegetative organs before anthesis and then translocated to the grains constitutes the grains' main source of N (Spiertz and De Vos, 1983; Van Sanford and MacKown, 1987), amounting to 50-95% of their total N at harvest (Palta and Fillery, 1993; Kichey et al., 2007). Nitrogen uptake after anthesis can increase the grain N content significantly (Mi et al., 2000; Woolfolk et al., 2002; Bly and Woodward, 2003), but post-anthesis N uptake is strongly dependent on amount and distribution of spring rainfall, rendering this contribution to grain N unreliable in Mediterranean environments.

GNO is generally considered as representative of the sink for grain nitrogen (Martre et al., 2006), but when the source-sink approach is used to explain genotypic variability in grain N, grain weight should also be considered, as grains are not just boxes of carbon, but they must also acquire N in appropriate proportions to their weight as they grow (Mi et al., 2000). A large genotypic variability exists in durum wheat for grain weight (Motzo et al., 2015; Giunta et al., 2019), and grain weight is an even more 'conservative' trait than GNO (Sadras and Slafer, 2012). Therefore, in Mediterranean environments, grain N content strongly depends on the source-sink ratio established at anthesis between the number and potential size of grains set and the amount of N absorbed by this stage.

The amount of N absorbed by the crop is related to its biomass (Lemaire et al., 2008). This means that differences in biomass at anthesis also contribute towards the differences in the N source available to the grains. At the same time, biomass at anthesis is also associated with sink strength, at least when modern cultivars sharing a similar biomass partitioning between spikes and total biomass are compared (Fischer, 2011). More precisely, both GNO and grain weight potential (Calderini et al., 2001) depend on the assimilates available in the 20–30 days around anthesis (Fischer, 2011). N uptake and biomass at anthesis can therefore be considered as representative of the source-sink strength for grain N accumulation in post-anthesis.

The aim of the present work was to identify the genotypic constraints to the realization of high grain yields with high grain N contents by analysing the traits responsible for grain yield and grain N in cultivars with contrasting phenotypes for these traits. Four cultivars were carefully selected by analyzing data from the 'National Italian Network for durum wheat cultivar comparison' to represent a continuous range of variation in grain yield and grain N: cultivar Aureo, characterized by consistently high grain protein percentages and low grain yields; cultivars Iride and Ramirez, characterized by low grain protein percentages and medium to high grain yields; cultivar Svevo with an intermediate performance, due to a grain yield comparable with Iride and Ramirez in some seasons, and a grain protein percentage higher than Iride and Ramirez but lower than Aureo (Pruneddu et al., 2012-2015). These cultivars were compared in field experiments under three N rates in a Mediterranean environment, and their grain yields and grain N were analyzed with particular emphasis on GNO determination, the N source and sink and the effects of the varying N rates on these traits.

2. Materials and methods

The study was carried out for the seasons 2015/16 ('2016'), 2016/17 ('2017') and 2017/18 ('2018') at the experimental station in Oristano (40° N; 8° E; 15 m altitude) belonging to the University of Sassari. The soil was a clay loam with a depth of about 2 m. The average soil water content in the first 2 m layer on a volumetric basis was 38% at field capacity (-0.02 MPa), and 19% at the permanent wilting point (-1.5 MPa). Mineral soil N amounted to about 50 kg ha^{-1} in the 0–2 m soil layer, and the soil organic matter of the first 0.8 m was 1.0%. The locate climate is typically Mediterranean, with a long-term average annual rainfall of 575±139 mm, mainly occurring between October and April.

The three seasons were characterized by very different weather conditions: the total amount of rainfall for the October–June period was 748 mm in 2018, which was more than double the amount recorded in the other two seasons and 42% greater than the long-term average (61 years, same site) (**Fig. 1**). The spring rainfall of 2018 was particularly abundant due to an exceptionally rainy May (230 mm vs 24 mm in 2016 and no rain at all in 2017). The lowest seasonal rainfall was recorded in 2017, when only 234 mm fell between October and June, equivalent to 44% of the long-term average. The month of December was unusually dry in both 2016 and 2017, as highlighted by rainfall that was lower than the cumulated reference evapotranspiration (by 10 and 28 mm, respectively). The seasonal pattern in the fraction of extractable water in the soil layer explored by roots revealed a soil close to field capacity for the entire 2018 growing season, with frequent periods characterized by a FEW above 1, indicating that the soil was above field capacity also in the month of May, when most of the grain filling took place. In 2016 and 2017, the FEW was lower than in 2018, but still well above 0.55 until anthesis. Terminal water stress, typical of this type of environment, developed after anthesis in these two years. Winters were mild, with monthly average temperatures never falling below 4°C. In 2016, a sharp decrease in the daily minimum temperature was observed (from 12.5 to 1°C) between booting and anthesis, whereas spring minimum temperatures were higher in 2018

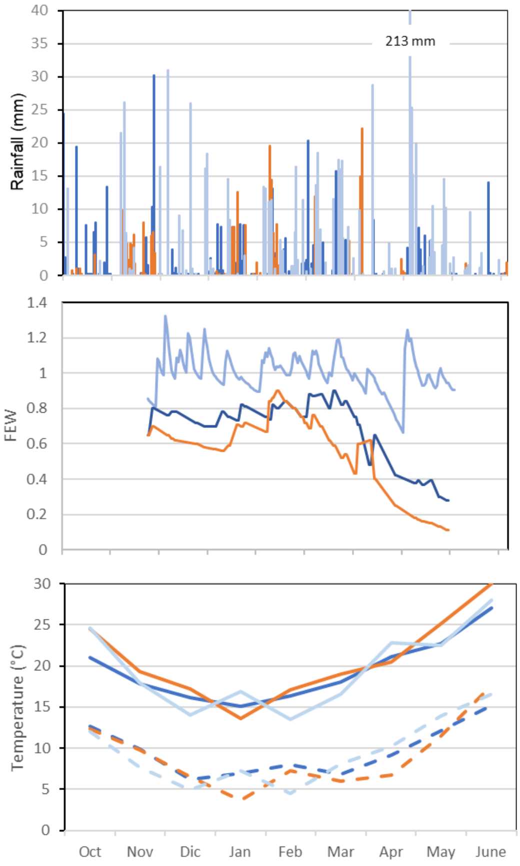

Figure 1. *Rainfall and ETo, Fraction of Extractable Water (FEW) in the soil layer explored by roots, and maximum and minimum (dashed lines) temperatures from October to June for the seasons 2016 (blue), 2017 (orange) and 2018 (light blue).*

The seed-bed was prepared by chisel-ploughing to a depth of 0.25 m, followed by surface cultivations. Sowing was performed on 19 November 2015, 23 November 2016 and 23 November 2017 at a rate of 350 viable seeds m^{-2} with a 9-row planter. Each plot consisted of 9 rows, 0.13 m apart and 30 m long, covering a total area of 35 m^{2}. In all cases, the preceding crop was a grain legume (faba bean or lupin). Nitrogenous and phosphorous fertilizers were applied as urea and triple superphosphate. Phosphorous was applied at sowing as P_2O_5 at a rate of 92 kg ha^{-1}; N was applied according to the treatments described below. Plots were kept free of weeds, pests and diseases using the appropriate chemicals.

2.1. Treatments and design

The modern Italian durum wheat (*Triticum turgidum* L. subsp. *durum* Desf.) cultivars compared in this experiment were selected on the basis of the data obtained from the 'Italian National Network for cultivar comparison'. Those selected were characterized by a continuous range of variation in grain yield and grain protein percentage. The data analyzed regarded four seasons (2011/12, 2012/13, 2013/14 and 2014/15) and two locations (Oristano and Sassari) characterized by the same type of Mediterranean environment as analyzed here (**Fig. S1**). The four cultivars selected for the present experiment based on their mean grain protein percentage and grain yield were: Aureo, characterized by high grain protein percentage (13.5±1.1; mean ± standard deviation) and low grain yield (6.0±1.0); Iride and Ramirez, which combined a low grain protein percentage (11.3±0.87% and 11.2±0.72%, respectively) with a medium to high average grain yield (8.2±1.7 and 7.8±1.5 t ha^{-1}, respectively); and Svevo with intermediate grain protein percentage (12.3±0.7%) and grain yield 6.8±1.5 t ha^{-1} (Pruneddu et al., 2012-2015).

The four cultivars were compared at three nitrogen fertilization rates (**Tab. 1**): treatment 'N0' received no N fertilization; treatment 'N80' received a total of 80 kg N ha^{-1} split between a first application as top dressing at sowing and a second application at the end of tillering/onset of stem elongation; treatment N160 received a total of 160 kg N ha^{-1}, split between sowing (80 kg ha^{-1}), onset of stem elongation, and booting (40 kg ha^{-1} each).

Table 1. Sowing dates and N treatments applied

Stage	2016	2017	2018	Kg N ha^{-1}		
				N0	N80	N160
Sowing	19 November	23 November	23 November	0	40	80
Stem elongation	18 January	3 February	1 February	0	40	40
Booting	15 March	24 March	30 March	0	0	40

Treatments (cultivars and nitrogen rates) were arranged in a split-plot design with three replications. Cultivars were assigned to the main plots and nitrogen treatments to the sub-plots.

2.2. Measurements

The following stages were recorded by inspecting the plots periodically (twice a week): onset of stem elongation (DC 31, Zadoks et al., 1974); booting with first awns visible (DC 49); anthesis (DC 61); dough maturity (DC 85); and physiological maturity (DC 92).

In order to determine the biomass and N accumulated by the crops, uprooted plant samples from a 0.26 m^2 area were taken from the central rows of each plot at anthesis, dough stage and harvest. Anthesis and dough maturity samples were divided into 'leaves' (leaf laminas), 'stems' (true stems plus leaf sheaths) and 'ears'; the harvest samples were divided into straw and grain. All biomass samples and sub-samples were oven-dried at 80°C for 48 hours before weighing. Nitrogen percentage was determined for each biomass subsample by means of a Carbon/Hydrogen/Nitrogen Analyzer (628 Series, LECO Corporation, St. Joseph, MI, USA) and used to obtain the amount of N allocated to each biomass subsample per unit surface. The nitrogen harvest index (NHI) was then calculated as the ratio between the N allocated to grains and the total N present in the above-ground biomass at maturity. The amount of N taken up after anthesis was evaluated as the difference between total N per unit surface at maturity and total N per unit surface at anthesis. N translocated to the grains was roughly estimated as the decrease in N of vegetative organs between anthesis and maturity and expressed as the percentage of the N present in the grains at maturity.

Critical N was calculated using the critical N dilution curve for wheat described by Justes et al. (1994) and used to determine the Nitrogen Nutrition Index (NNI) at anthesis as the ratio between the actual above-ground crop N percentage and critical N. The NNI of the ear (earNNI) was also calculated at dough maturity using the critical ear N dilution curve proposed for wheat ears by Zhao et al. (2020) as the ratio between the actual ear N percentage and ear critical N.

The harvest index (HI) was determined for the 'maturity' samples and used to calculate the final above-ground biomass from the grain yield obtained on a plot basis with mechanical harvesting. Grain weight was assessed as the mean of four 250 grain sub-samples per plot. The grain moisture content was used to express both grain yield and grain weight on a 0% moisture basis. The number of grains per m^2 (GNO) was calculated by dividing grain yield by grain weight. The mg of N present in each grain were calculated by multiplying grain weight by grain N percentage. Grain protein percentage was calculated as grain N percentage by 5.7.

Weather data (maximum and minimum temperature, rainfall, solar radiation and air relative humidity) were recorded in a meteorological station located approx. 300 m from the field and used to calculate the reference evapotranspiration.

The SunScan Canopy Analysis System SS1-UM-1.05 (Delta-T Devices) was used to measure the photosynthetically active radiation (PAR) intercepted by the canopy during the central hours of the day at each biomass sampling. Mean PAR at the soil level was assessed by aligning the probe at right angles to the row direction and parallel to the soil surface at three different points along the plot. The probe was positioned at ground level. At the same time, the Beam Fraction Sensor monitored the light incident at the canopy surface. The fraction of intercepted radiation, calculated as the ratio between the differences in incident and transmitted to incident radiation, was used to calibrate canopy cover in the AquaCrop model (Steduto et al., 2012). Canopy cover, weather, soil and management data were used to simulate the soil water balance with Aquacrop. The soil water content in the soil layer explored by roots simulated by the model was expressed as the fraction of extractable water (FEW), i.e. the difference between the soil water content at field capacity and the actual soil water content, divided by the available water.

2.3. Statistical analysis

After assessing the homogeneity of variances using the Bartlett Test, combined analysis of variance (ANOVA) was performed, superimposing the year as the main plot on the original design (Gomez and Gomez, 1984). In the resulting split-split-plot design, year was the whole plot factor, cultivar was the sub-plot factor, and fertilization treatment was the sub-sub-plot factor. Year was considered as a fixed factor because the sampled years were very different in rainfall amount and distribution and generated interesting interactions with both cultivars and N treatments. Statistical analyses were conducted using R software (R Core Team, 2017), package 'agricolae', ssp.plot procedure. Following a significant F test, means were compared using the least significant difference (LSD) test, and considering a 0.05 probability level as statistically significant. The appropriate standard errors and t values were used for each type of comparison (Gomez and Gomez, 1984). The Pearson's correlation coefficient was calculated to evaluate the existence of any causal relationships between pairs of traits.

3. RESULTS

Both main effects and interactions were significant as assessed by ANOVA for most of the considered traits. Therefore, interactions are presented as Figures, while main effects and ANOVA results are available as Supplemental Tables.

Cultivars Aureo, Svevo and Iride were almost synchronous with regard to the average anthesis date (2 April 2) and dough maturity date (10 - 12 May). Ramirez was slightly later, reaching each stage about 4 days after the other cultivars (**Tab. S1**). The fertilization treatment did not affect booting or anthesis dates.

3.2. N uptake by anthesis

An overall increase in total N was observed as the N rate increased, but the cultivar Aureo displayed the greatest increase, in particular to the highest fertilization rate: about 250 kg of N ha^{-1} accumulated in the biomass of cultivar Aureo at N160 in 2017 and 2018 against values ≤ 200 kg ha^{-1} in the other cultivars (**Fig. 2**). Cultivar Aureo was characterized by its particularly high N percentage and N content in stems and high N content in leaves per square meter (**Tab. S2**) at anthesis. The amount of N available in each stem by anthesis in this cultivar (20.3 mg stem^{-1}) was almost double the amount available in the stems of cultivar Ramirez (11.1 mg stem^{-1}) (**Tab. S2**).

Cultivar Aureo also had the best N nutritional status at anthesis compared with all the other cultivars for all the year and N treatment combinations which allowed for the expression of the cultivar differences. Aureo was the only cultivar in the N160 treatment with an NNI ≥ 1 at anthesis in both 2016 and 2018. An opposite combination of traits was recorded in cultivar Iride.

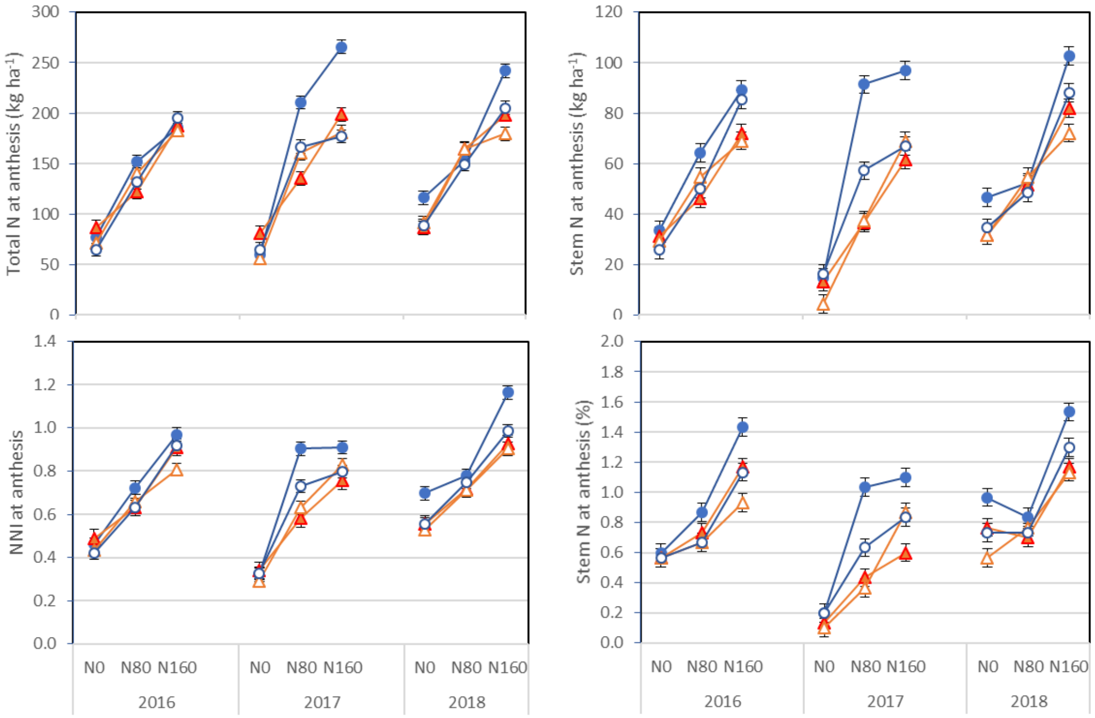

Figure 2. *N uptake by anthesis and Nitrogen Nutritional Index (NNI) at anthesis. Cultivar x year x N treatment means. Aureo, full blue circles; Svevo, empty blue circles; Iride, full orange triangles; Ramirez, empty orange triangles. Bars represent standard errors of the means.*

3.3. Post-anthesis N uptake and allocation

The N nutritional status after anthesis, evaluated by determining the earNNI at the dough-maturity stage, highlighted the ability of the N160 treatment to guarantee an optimal N nutritional status of the ear for all the cultivars (**Tab. S3**)

N treatment was also effective in changing the total N accumulated by maturity for almost all the cultivars and years (**Fig. 3**).

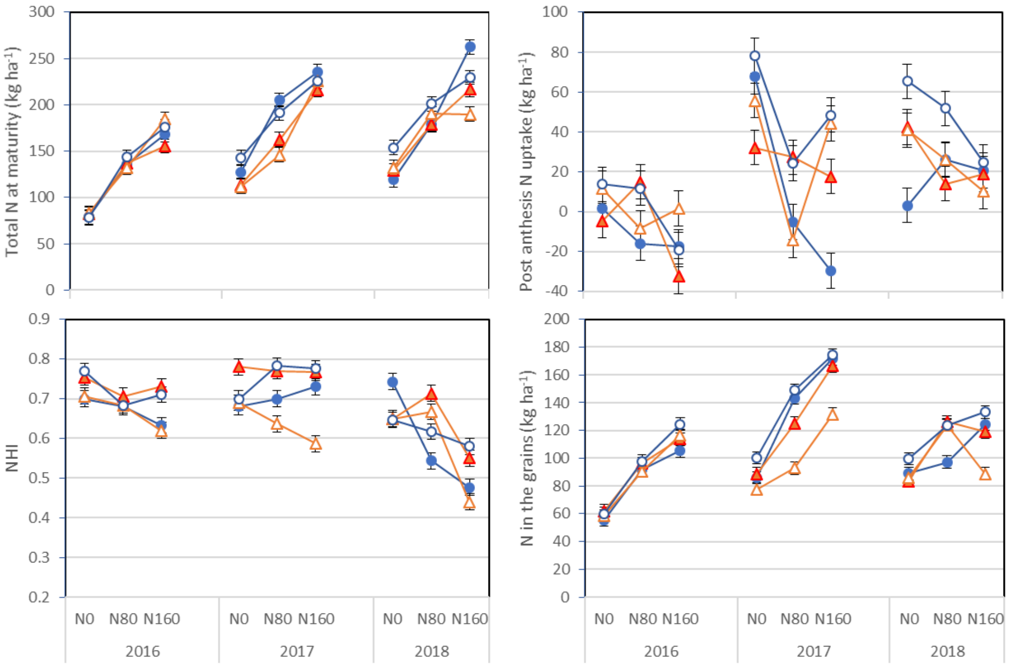

__Figure 3__. Total and grain N at maturity, N partitioning (Nitrogen Harvest Index) and post-anthesis N uptake. Cultivar x year x N treatment means. Aureo, full blue circles; Svevo empty blue circles; Iride, full orange triangles; Ramirez, empty orange triangles. Bars represent standard error of the means.

Aureo and Svevo were the cultivars with more N in their biomass at maturity on average (170 kg ha^{-1} in Aureo and Svevo vs 155 kg ha^{-1} in Iride and Ramirez), although cultivar differences in total N were only expressed in 2017 and 2018. Post-anthesis N uptake showed a strong cultivar x N treatment interaction, but was generally higher at N0 than at N180, a result that can only be explained by admitting that a great part of the last N application, made at booting, was taken up before anthesis, and/or, that N losses occurred between anthesis and maturity.

In the case of Aureo, its higher total N at maturity was mainly a consequence of its higher N uptake by anthesis, which was also associated with a lower average post-anthesis N uptake (6 kg ha^{-1} against an average of 22 kg ha^{-1} in the other three cultivars). As a consequence, Aureo displayed the greatest relocation of N from vegetative organs to the grains (93% against an average of 79% in the other three cultivars **Tab. S3**). By contrast, the high total N observed in Svevo at maturity was attributed to its opposite combination of traits, i.e. a greater post-anthesis N uptake, particularly in 2018, and a lower average N relocation to the grain compared with all the other cultivars (71%, **Tab. S3**).

According to their NHI, Aureo and Ramirez allocated a smaller proportion of their total N to the grains compared with cultivars Iride and Svevo, particularly at N80 and N160 in seasons 2017 and 2018. Aureo and Ramirez were also the cultivars with a lower HI (0.32 in Aureo and 0.35 in Ramirez on average, against values above 0.37 in the other two cultivars, **Tab. S3**).

The total N accumulated in the grains as a consequence of the combination of total N uptake and NHI was maximal in cultivar Svevo in almost all the year x N treatment combinations (118 kg N ha^{-1} on average), whereas it was lower and similar in cultivars Aureo and Iride (107 and 109 kg N ha^{-1}, respectively).

3.4. Grain yield and grain protein

Compared with the other seasons, 2017 had the highest mean grain yield (7.44 t ha^{-1}) The grain yield in 2018 was likely penalized by the lower grain weight derived from both excessive rainfall and high minimum temperatures during grain filling, whereas the low minimum temperatures in 2016 between heading and anthesis resulted in very low ear fertility and GNO. Nitrogen fertilization up to N160 was effective in increasing (relative to N0 values) both grain N content (from 0.68 to 0.95 mg N grain^{-1} on average) and grain protein percentage (from 8.8 to 14.0% on average) (**Fig. 4**).

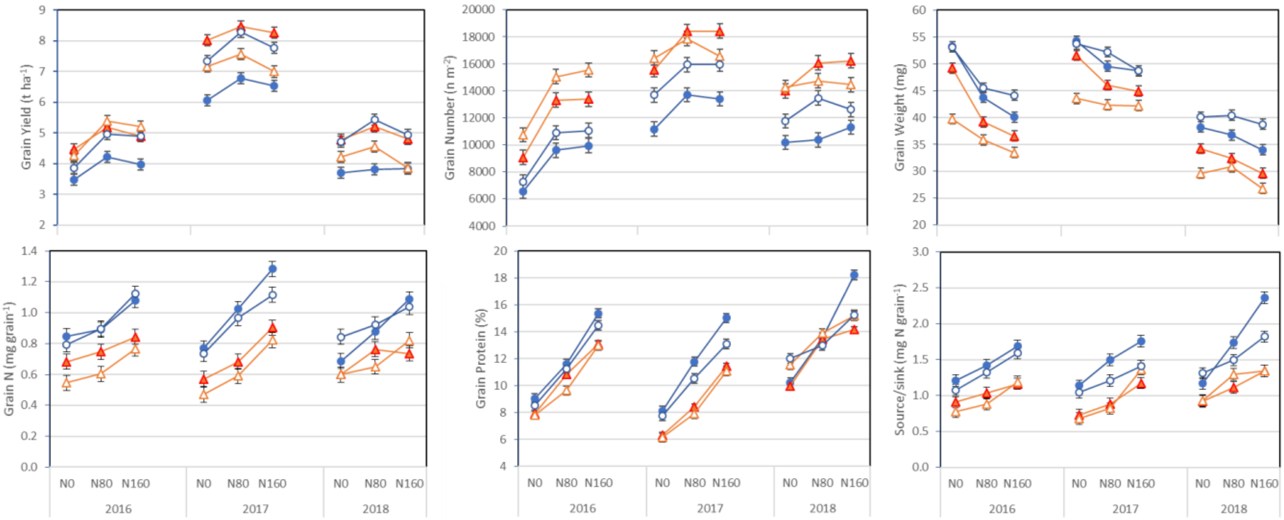

Figure 4. Grain yield and grain yield components, grain N content and protein percentage, Nsource (total N): Nsink (GNO) ratio. Cultivar x year x N treatment means. Aureo, full blue circles; Svevo empty blue circles; Iride, full orange triangles; Ramirez, empty orange triangles. Bars represent standard error of the means.

In almost all the year x N treatment combinations shown in Fig. 4, cultivars Aureo and Svevo combined lower GNO with higher grain weights, grain N contents and protein percentages. This contrasts with the combination of traits observed in cultivars Iride and Ramirez. The lower GNO combined with the higher total N taken up by maturity generated a greater availability of N per grain, equal to 1.55 and 1.36 mg N grain^{-1} in cultivars Aureo and Svevo, respectively, compared with 1.03 mg grain^{-1} in the other two cultivars. These results are in line with the observed differences in grain N content (**Fig. 4, Tab. S4**). The potential grain weight of the four cultivars was also calculated as the maximum grain weight across the 27 means recorded in the different combinations of environmental conditions and N treatments generated by this experiment and the data-set of the Italian National Network for durum wheat cultivar comparison, years 2012-2020 (**Fig. S2**). The rank in potential grain weight was the same of the rank in mean grain weight: Svevo was the cultivar with the highest potential grain weight (59.8 ± 1.1 mg, maximum ± standard error of the mean), followed by Aureo (57.3 ± 1.2 mg), Iride (56.5 ± 1.4 mg), and Ramirez (47.4 ± 1.1 mg).

By contrast, the cultivar ranking with regard to grain yield varied according to year and N treatment. The only consistent position across Y x N interactions was Aureo's low grain yield. Aureo was also the cultivar showing the largest increase in grain protein percentage in 2018, whereas the other three cultivars benefited less from the raise in N rate from N0 to N160.

The variation in GNO, which was partly responsible for the variation in the amount of N available per grain, was analyzed by its numerical components ears m^{-2} and grains ear^{-1}, once a lack of any compensation/trade-offs between ears m^{-2} and number of grains per ear had been ascertained via a correlation analysis. The variation in ear fertility (number of grains per ear) was the only variable contributing to the between-year variation in GNO, whereas both components reacted to N treatment in the same way as GNO, increasing from N0 to N80 only. The lower average GNOs for cultivars Aureo and Svevo compared with those for cultivars Iride and Ramirez derived from their inability to set a number of ears m^{-2} comparable to cultivar Ramirez and/or a number of grains per ear as high as cultivar Iride.

These results were further corroborated by the data gathered by the Italian network for cultivar comparison for the four years used to select the four cultivars, plus further 5 years available since then at 2 sites (Pruneddu et al., 2012-2020 (**Fig. 5**). One of the sites was the same as used in this experiment (Oristano), the other was Sassari, located approximately 100 km to the north.

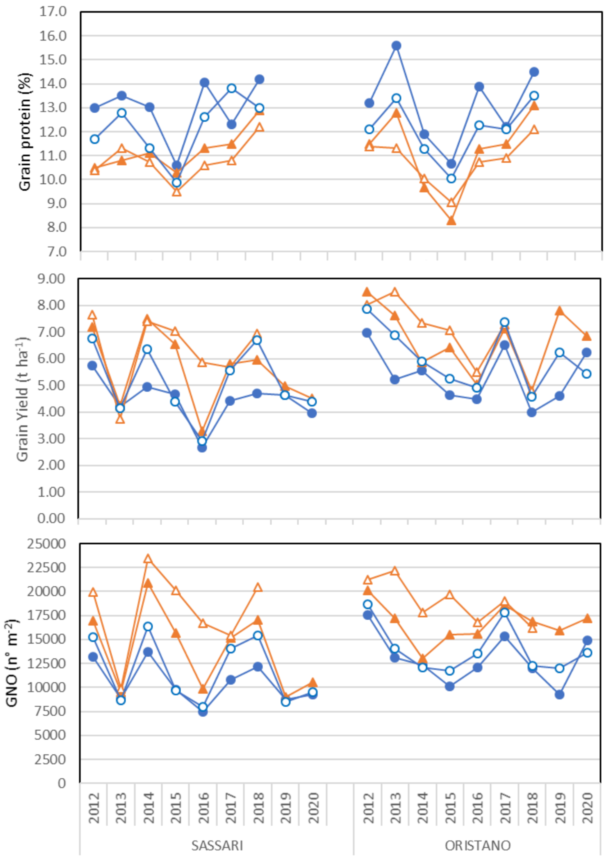

Figure 5*. Inter-annual variation (from 2012 to 2020) in grain protein percentage, grain yield and grain number recorded by the Italian National Network for durum wheat cultivar comparison for the four cultivars analyzed in this experiment at the sites Oristano and Sassari (Pruneddu et al., 2012-2020). Aureo, full blue circles; Svevo empty blue circles; Iride, full orange triangles; Ramirez, empty orange triangles. Grain protein data for the 2019 and 2020 seasons and Ramirez data for the same two seasons were not available.*

These data show cultivars Aureo and Svevo to exhibit consistently higher grain protein percentages compared with Iride and Ramirez for a grain yield range similar to that explored in our experiment. They also confirm the ability of cultivar Svevo, but not cultivar Aureo, to produce grain yields comparable to those of Iride and Ramirez, as well as yields greater than 5.5–6.0 t ha^{-1}, as obtained in 2012, 2014, 2017 and 2018 at Sassari, and 2012, 2013 and 2017 at Oristano, which were partly due to GNOs greater than 15000 (the only exception was Oristano, 2013).

The relationships between grain protein percentage and NNI at anthesis, earNNI and total N at anthesis were calculated for each cultivar to highlight any difference between cultivars and to identify the best predictor of the grain protein percentage. As no difference was detected in slope and intercept between cultivars, unique relationships were calculated using the C x Y x N means. The C x Y x N variation in grain protein percentage at maturity was more strongly associated with plant NNI at anthesis (r = 0.89***, n = 36, **Fig. 6**) and with earNNI at the dough maturity stage (r = 0.92***, n = 36, **Fig. 3S**) than with the total N taken up by anthesis (r = 0.80***, n = 36, **Fig. 3S**) or with the total N present in the crops at maturity (r = 0.77***, n= 36, **Fig. 3S**).

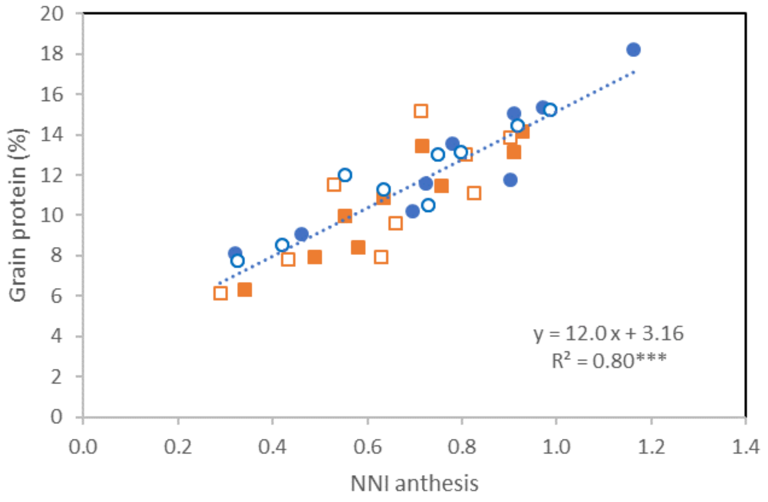

Figure 6*. Relationship between N nutritional status at anthesis expressed in terms of Nitrogen Nutritional Index and grain protein percentage. Data are cultivar x year x N treatment means. Aureo, full blue circles; Svevo, empty blue circles; Iride, full orange squares; Ramirez, empty orange squares. ***, significant at P ≤ 0.001.*

4. Discussion

Much of the interest and utility of this experiment in explaining the genotypic constraints to the realization of high grain yields with high grain protein percentage in durum wheat relied on the cultivars used, because of the large range in the relationship between grain protein percentage and grain yield they expressed. At the same time, the minimal variation in anthesis date between cultivars avoided the confounding effect of different developmental rates, placed the critical period for grain number determination of the four cultivars under the same environmental conditions, and assured a similar duration of the post-anthesis period.

Moreover, the genotypic base of high grain protein percentage of cultivars Aureo and Svevo over Iride and Ramirez and their inferiority in GNO was sustained by its being highly consistent across years, N treatments and yield levels (from 3.5 to 8.5 t ha^{-1}), despite the large variation in environmental conditions sampled with this experiment. The 2016, 2017 and 2018 seasons presented extreme conditions for this type of Mediterranean environment in terms of rainfall amount and distribution, and the N treatments covered the widest range in N fertilization possible for this type of environment. N80 (40 kg N ha^{-1} at sowing plus 40 N ha^{-1} at the onset of stem elongation) is the most common fertilization practice adopted for durum wheat in this type of Mediterranean environment. The N160 treatment was planned in order to favour both vegetative growth and N accumulation in grains: of the extra 80 kg of N ha^{-1} (compared with N80 treatment), half were applied at sowing, and half were applied at booting, when N fertilization mainly affects grain N content (Fischer et al., 1993; Gaju et al., 2014). The importance of the genotype x management interaction for both grain yield and grain protein percentage has been highlighted by Cooper et al. (2001), and N fertilization is perhaps the management practice that affects the most the two traits considered and their relationship.

An unexpected result linked to the N treatment was the generally higher post-anthesis N uptake at N0 than at N160, in spite of the late third N application made at N160. It can be explained by admitting that a great part of this last N application was taken up between booting (awns visible) and anthesis, but also assuming that N losses occurred between anthesis and maturity. Plants can release N from the senescing leaves into the atmosphere (Farquhar et al., 1983), and from roots into the soil, thus reducing the final N content to 60-80% of the N present at heading (Daigger et al., 1976; Denmead et al., 1974). These losses are particularly likely when the total N present in the biomass at anthesis is greater than 200 kg ha^{-1} (Papakosta and Gagianas, 1991; Cadeddu et al., 2021) and evapotranspiration rates are high after anthesis, such as in Mediterranean environments (Ehdaie and Waines, 2001).

The higher amount of N in the grain obtained from cultivars Aureo and Svevo in the present experiment, expressed as either protein percentage or mg of N per grain, was associated with both a greater N source, defined here as the N absorbed by anthesis plus the post-anthesis N uptake, and a smaller sink, in terms number of grains per unit surface. Potential grain weight, on the contrary, the other component of the N sink, was larger in cultivars Aureo and Svevo.

The association of high grain protein and high grain weight found in cultivars Aureo and Svevo is only in apparent contrast with the negative association often quoted in the literature between these two traits. Negative associations between grain weight and grain protein usually derive from environmental, not genotypic variation, because the large grains produced in the more favourable seasons are responsible for the so-called 'dilution effect' associated with low grain protein percentage (Sander et al., 1987). Conversely, the genotypic variation in bread and durum wheat grain weight was demonstrated to be associated to the genotypic variation in grain filling rate (Robert et al., 2001; Motzo et al., 1996; Motzo et al., 2010) and the latter to the N accumulation rate (Robert et al., 2001; Giunta and Motzo, 2005), suggesting that cultivars with high grain weights are expected to display also high grain N contents and percentages.

The hypothesis that the low GNO obtained by Aureo and Svevo is derived from inadequate N fertilization and/or a low nutritional status could be rejected by the lack of any increase in GNO with a rise in fertilization rate from N80 to N160 despite the improved NNI and N uptake. In fact, no relationship was found between NNI at anthesis and GNO (**Fig. 4S**), in contrast with what proposed by Zhang et al. (2021). The discrepancy between our results and those of Zhang et al. (2021) could be a consequence of the different range explored in both GNO (greater than 20000 grains m^{-2} in their paper, lower than 20000 grains m^{-2} in our experiment) and in NNI (between 0.7 and 1.0 in their paper, and between 0.3 and 1.0 in our data). GNO is determined during a rather limited period of time: from the appearance of the penultimate leaf until anthesis (Fischer, 2011). In our experiment, the two cultivars with the lower GNO, Aureo in particular, were also characterized by a higher N content and percentage in the stems and ears at anthesis. Indeed, these results suggest a negative effect of a high N content and percentage on GNO determination, which could be explained on the basis of the positive role of carbohydrate availability on floret survival, as discussed by several different authors, each of whom employed a different experimental approach (Boyer and McLaughlin, 2007; Ghiglione et al., 2008; Dreccer et al., 2014). Although we did not measure carbohydrate levels at anthesis, it is well established that the variation in shoot N concentration induced by a variation in N fertilization rate correlates negatively with water-soluble-carbohydrates accumulation (Bänziger et al., 1994, van Herwaarden et al., 1998, Tahir and Nakata, 2005). One explanation proposed for this observation is that a common pool of reduced carbon and energy is used to produce both nitrogen compounds and water-soluble-carbohydrates (van Herwaarden et al., 1998, McIntyre et al., 2011), leading to a reduction in water-soluble-carbohydrates accumulation under high concentrations of N. The same reasoning could be applied when genotypic variation is involved, as a large genotypic variation in water-soluble carbohydrates has been reported (Foulkes et al.,2002; Ehdaie et al., 2006). Thus, a lower carbohydrate availability could be hypothesized in cultivars Aureo and Svevo based on the higher energetic costs sustained to build stems and ears with a higher N percentage in comparison with the other two cultivars. The negative relationship calculated between GNO and N percentage in stems presented in **Fig. 7** is consistent with this hypothesis, and could help explain the low GNO of the two cultivars with the higher grain N content and percentage. This relationship was not expressed at the lower N rate, and its strength increased progressively at increasing N rate, likely because of the corresponding increase in N percentage in stems and hence in the potential higher energetic cost sustained by the crops to build their biomass. The uneven distribution of cultivar means – Aureo and Iride on opposite sides of the relationship - is indicative of a genotypic base of these relationships. In some way, our hypothesis fits to one of the main hypotheses proposed to explain the physiological basis of the negative correlation between grain yield and grain N content, i.e a competition between C and N for energy (Munier-Jolain and Salon, 2005).

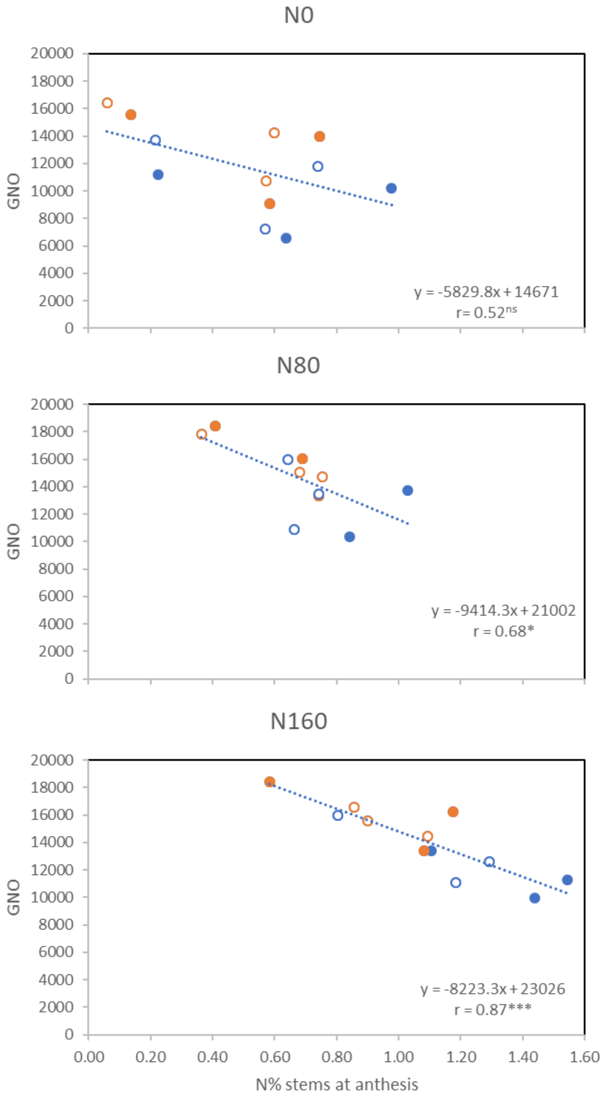

***Figure 7**. Negative effect of N percentage in stems at anthesis on GNO determination at the different N rates. Aureo, full blue circles; Svevo empty blue circles; Iride, full orange circles; Ramirez, empty orange circles.*

Our hypothesis is in apparent contrast with Ferrante et al. (2013), which postulated a positive role of N availability on number of grains in durum wheat. In that case the positive effect of N availability was evaluated within cultivars and no information was given on the N percentage in the shoot at anthesis. On the contrary, we are talking about the genotypic variation in stem N concentration at anthesis at similar N rates, highlighting how the ability of some cultivars, such as Aureo, to build a great N source by anthesis through very high N percentages in stems might have a negative impact on grain number. In the case of Prystupa et al. (2004), the strong positive relationship between spike N and grain number was not due to a variation in spike N concentration, and only reflected the variation in spike dry mass induced by varying N rate. Our hypothesis is also in contrast with *Zhang et al. (2021), who proposed a positive effect of pre-anthesis plant N status on potential ear N sink, because no association was found between NNI at anthesis and GNO.*

Grain N content is generally considered to be 'source limited' in modern wheat cultivars (Martre et al., 2006), and Iride and Ramirez, the cultivars with the lower grain N content, were characterized by a lower N source compared to Aureo and to Svevo, and by a moderate N deficiency, even at the higher N rate, meaning that they were not able to satisfy their own N demand.

A high source capacity (N at anthesis plus post-anthesis N uptake) was another distinctive trait of the two cultivars with a high grain protein percentage. However, the source capacity of Aureo was almost entirely due to pre-anthesis N uptake, whereas Svevo showed a greater post-anthesis N uptake.

The great difference observed in cultivar Aureo in its ability to absorb N before and after anthesis can be partly attributed to the above-mentioned post-anthesis N losses. Given the association between amount of N accumulated in the shoot at anthesis and the amount of post-anthesis N losses (Ehdaie and Waines, 2001), Aureo was the cultivar more prone to N losses after anthesis, particularly in 2017, when more than 250 kg N m^{-2} were accumulated in its shoot by anthesis. Moreover, its great pre-anthesis N uptake likely reduced the amount of N available in the soil after anthesis, and the extremely high NNI and N percentages recorded at anthesis in all its organs, stems in particular, likely inhibited post-anthesis N uptake (Glass, 2003). The consistent superiority of cultivar Aureo for these traits at anthesis confirms that genotypic variability exists for them, not only in bread (Gaju et al., 2014) but also in durum wheat. It is likely that the greater increase in grain protein percentage of cultivar Aureo compared with the other cultivars in response to increasing N fertilization rates was derived from this greater capacity to accumulate N in the vegetative organs. A high capacity to load N in stems (plus sheaths)—both on a unit surface basis and per single stem—is, therefore, a desirable trait for improved N uptake and relocation to grains, as long as it does not simply involve a greater amount of structural N (Pask et al., 2012).

As far as Svevo is concerned, its lower N uptake before anthesis compared to Aureo could be due to a lower ability to store excess N in vegetative tissues, as genotypic variability for this trait has been reported in wheat (Gaju et a., 2014). At the same time, the greater post anthesis N uptake of cultivar Svevo did not derive from an earlier anthesis and a longer post-anthesis period - a common cause of genotypic differences in post-anthesis N uptake (Giunta et al., 2020) – as Aureo and Svevo flowered almost contemporarily. The reason behind the greater post-anthesis N uptake of cultivar Svevo could instead be searched in its greater sink capacity compared to Aureo, due to both a higher GNO than Aureo and a higher potential grain weight (Mi et al., 2000). Post-anthesis N uptake can contribute between 5 to 50% of grain N (de Ruiter and Brooking, 1994) depending on soil N availability and environmental conditions. In Mediterranean environments, post-anthesis N uptake is strongly conditioned by the unpredictable and scarce availability of water in the spring; nevertheless, cultivar Svevo was able to take up almost the same total amount of N as cultivar Aureo in each of the three years of the experiment despite the differences in spring rainfall, and to combine high grain protein content with grain yields comparable to the grain yield of cultivar Iride and Ramirez. In fact, Bogart et al. (2010) suggested that the genotypic deviation from the negative relationship between grain yield and grain N could be related to post-anthesis N uptake independently of anthesis date and total N at anthesis under most environments, and that the physiological trait responsible for this genotypic difference is the ability to maintain root activity during the grain-filling period. The high seasonal and spring rainfall were the traits that distinguished the site x year combinations when cultivar Svevo realized grain yields comparable to Iride and Ramirez and associated to high N content in the data-set form the Italian network for cultivar comparison (Pruneddu et al., 2012-2020). This result suggests that the hypothesized greater/longer root activity after anthesis of cultivar Svevo also favoured grain filling and the realization of high grain weights.

The strong correlation between NNI at anthesis and grain protein percentage, although lacking a genotypic base, was an interesting result because it meant that the nutritional status at anthesis, as described by the NNI, was highly indicative of grain protein percentage at maturity, regardless of cultivar, year, N treatment, sink strength and post-anthesis N uptake. Justes et al. (1997) highlighted that the relationship between NNI at anthesis and grain protein percentage only holds when post-anthesis N uptake is below 55%, as in our experiment. The importance of the N taken up by anthesis as the major source of N for the grain is well recognized (Gaju et al., 2014), but its capacity to predict grain protein percentage in this experiment was greater when expressed as NNI rather than kg of N ha^{-1}. Being valid irrespective of the cultivar, the relationship between grain protein percentage and NNI at anthesis could represent a useful trait to select by anthesis lines/cultivars with high grain protein percentages. The earNNI—proposed by Zhao et al. (2020, 2021) as an indicator of post-anthesis N status for bread wheat—was in fact effective in describing the N status of the ear, as demonstrated by its association with grain protein percentage.

5. Conclusions

The aim of the present work was to identify the genotypic constraints to the realization of high grain yields with high grain N contents by analysing the traits responsible for grain yield and grain N in cultivars with contrasting phenotypes for these traits.

In Mediterranean environments durum wheat cultivars displaying a high grain N content and percentage also display poor ability to produce high GNOs, although they are not necessarily incapable of generating grain yields comparable to those of cultivars characterized by lower grain N content and percentages, at least in seasons characterized by favourable weather conditions when they can benefit from their high grain weight.

Two contrasting ‘strategies’ give rise to a high grain N content and percentage in such cultivars. The ‘strategy’ adopted by cultivar Aureo is based on its ability to build a large N source by anthesis. While this strategy consistently assures an extremely high grain N content, it is also extremely consistent in producing a low GNO, thus limiting Aureo’s potential to generate high grain yields. The ‘strategy’ characterizing cultivar Svevo, on the other hand, is based on its good capacity to absorb N after anthesis. Although this results in a grain N percentage that is lower and less reliable than that obtained by Aureo, it endows this cultivar with the ability to make the most of favourable weather conditions by combining a high grain N with a high grain yield.

The hypothesis that a high N concentration in stems and ears at anthesis could be the reason behind both a high grain N percentage and a low GNO deserves to be tested in future studies.

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Table S1. Main developmental stages.

	Booting	**Anthesis**	**Dough Maturity**
	(day of the year)		
Year	***	***	***
2016	80 c	91 b	126 c
2017	87 b	90 b	130 b
2018	94 a	97 a	140 a
Cultivar	***	***	***
Aureo	85 c	92 b	132 b
Svevo	85 c	92 b	130 c
Iride	87 b	92 b	132 b
Ramirez	91 a	96 a	136 a
N treatment	**ns**	**ns**	***
N0	87	93	131 c
N80	87	93	133 b
N160	88	93	134 a
C x Y	ns	ns	ns
C x N	ns	ns	ns
C x Y x N	ns	ns	ns

ns, F-test not significant at ANOVA, ***, F-Test significant at P≤0.001 Means with the same letter within each group of means are not statistically different at the LSD test for P≤0.05.

692

Table S2. Total biomass, N Nutritional Index, total N uptake and its partitioning to leaves (only laminas), stems (true stems plus leaf sheaths) and ears at anthesis.

	Total biomass (t ha^{-1})	Total N (kg ha^{-1})	NNI	Leaf N (kg ha^{-1})	Stem N (kg ha^{-1})	Stem N (mg stem^{-1})	Ear N (kg ha^{-1})	Leaf N (%)	Stem N (%)	Ear N (%)
Year	***	*	***	***	*	*	*	*	***	***
2016	9.9 b	133 b	0.68 b	46.2 b	54.2 b	15.6 ab	32.7 b	2.6 b	0.8 b	1.8 b
2017	12.8 a	146 a	0.59 c	63.8 a	47.1 c	13.5 b	35.5 a	2.6 b	0.5 c	1.6 c
2018	9.9 b	153 a	0.76 a	62.2 a	58.0 a	16.8 a	33.0 b	2.8 a	0.9 a	1.9 a
Cultivar	***	***	***	**	***	***	***	*ns*	***	***
Aureo	10.4 b	162 a	0.76 a	59.3 a	65.8 a	20.3 a	37.3 a	2.7	1.0 a	1.8 a
Svevo	10.6 b	138 b	0.68 b	54.5 c	52.5 b	15.5 b	31.0 b	2.7	0.8 b	1.7 b
Iride	11.4 a	140 b	0.64 c	55.5 c	47.3 c	14.3 b	37.3 a	2.6	0.7 c	1.6 b
Ramirez	11.1 a	136 b	0.63 c	60.2 a	46.9 c	11.1 c	29.3 b	2.7	0.7 c	1.8 a
Nitrogen	***	***	***	***	***	***	***	***	***	***
N0	8.7 b	79 c	0.45 c	31.0 c	26.0 c	8.5 c	21.7 c	2.2 c	0.5 c	1.5 c
N80	12.2 a	154 b	0.68 b	63.3 b	53.7 b	14.9 b	37.2 b	2.7 b	0.7 b	1.7 b
N160	11.8 a	200 a	0.89 a	77.9 a	79.6 a	22.5 a	42.4 a	3.1 a	1.1 a	2.0 a
Cultivar x Year	***	***	**	*	**	***	***	*ns*	*	**
Cultivar x N	***	***	***	***	***	***	***	*ns*	**	***
C x Y x N	***	***	***	***	***	***	***	*	***	*

Results of the F-test: ns, not significant; *, significant at P≤0.05; **, significant at P≤0.01; ***, significant at P≤0.001. Means with the same letter within each group of means are not statistically different at the LSD Test at P≤0.05.

Table S3. Total N, total biomass and their partitioning at maturity, post-anthesis N uptake and N Nutritional Index of the ear.

	Total biomass (t ha^{-1})	HI	Total N (kg ha^{-1})	NHI	N grain (kg ha^{-1})	Post-anthesis N uptake (kg ha^{-1})	N relocation (%)	NNIear
Year	***	***	***	*	***	***	***	***
2016	12.9 c	0.36 b	130 b	0.70 a	89 c	-3.5 b	102 a	0.87 b
2017	19.0 a	0.39 a	175 a	0.72 a	126 a	28.7 a	71 b	0.81 c
2018	14.1 a	0.32 c	182 a	0.61 b	108 b	28.7 a	74 b	0.98 a
Cultivar	**	***	***	**	***	***	***	***
Aureo	14.7 c	0.32 c	168 a	0.65 b	107 b	5.7 c	93 a	0.90 b
Svevo	15.9 a	0.37 b	171 a	0.70 a	118 a	33.2 a	71 c	0.94 a
Iride	15.4 ab	0.39 a	155 b	0.71 a	109 b	14.5 b	86 ab	0.85 c
Ramirez	15.3 bc	0.35 b	155 b	0.63 b	96 c	18.4 b	81 b	0.85 c
Nitrogen	***	***	***	***	***	***	***	***
N0	14.1 b	0.37 a	113 c	0.71 a	79 c	34.0 a	61 b	0.69 c
N80	15.9 a	0.37 a	1067 b	0.68 a	113 b	12.6 b	91 a	0.86 b
N160	15.9 a	0.34 b	207 a	0.63 b	131 a	7.3 b	96 a	1.10 a
Cultivar x Year	***	***	*	**	***	ns	ns	***
Cultivar x Nitrogen	*	**	*	**	**	*	***	***
C x Y x N	ns	ns	***	***	***	***	***	***

Results of the F-test: ns, not significant; *, significant at P≤0.05; **, significant at P≤0.01; ***, significant at P≤0.001. Means with the same letter within each group of means are not statistically different at the LSD Test at P≤0.05.

Table S4. Grain nitrogen content, grain yield and grain yield components

	Grain nitrogen (%)	Grain nitrogen (mg)	Grain yield (t ha^{-1})	Grain weight (mg)	Grains per unit area (n° m^{-2})	Ears per unit area (n° m^{-2})	Grains ear^{-1} (n°)
Year (Y)	***	ns	***	***	***	ns	***
2016	1.94 b	0.82	4.57 b	42.8 b	11036 c	366	33.1 c
2017	1.72 c	0.83	7.44 a	48.1 a	15559 a	347	48.1 a
2018	2.35 a	0.80	4.49 b	34.3 c	13290 b	351	36.8 b
Cultivar (C)	***	***	***	***	***	***	***
Aureo	2.20 a	0.95 a	4.71 d	44.3 b	10692 c	317 c	39.9 b
Svevo	2.06 b	0.94 a	5.80 b	46.3 a	12522 b	341 b	38.0 c
Iride	1.88 c	0.65 c	6.01 a	40.4 c	14939 a	330 bc	43.2 a
Ramirez	1.87 c	0.73 b	5.47 b	36.1 d	15071 a	432 a	36.3 c
Nitrogen (N)	***	***	***	***	***	***	***
N0	1.54 c	0.68 c	5.18 c	45.0 a	11726 b	328 b	37.1 b
N80	2.01 b	0.82 b	5.82 a	41.2 b	14119 a	363 a	40.1 a
N160	2.46 a	0.95 a	5.49 b	39.0 c	14074 a	373 a	40.9 a
C x Y	***	***	***	**	**	ns	ns
C x N	***	***	ns	***	ns	ns	ns
C x Y x N	***	***	ns	ns	ns	ns	ns

Results of the F-test: ns, not significant; *, significant at P≤0.05; **, significant at P≤0.01; ***, significant at P≤0.001. Means with the same letter within each group of means are not statistically different at the LSD Test at P≤0.05.

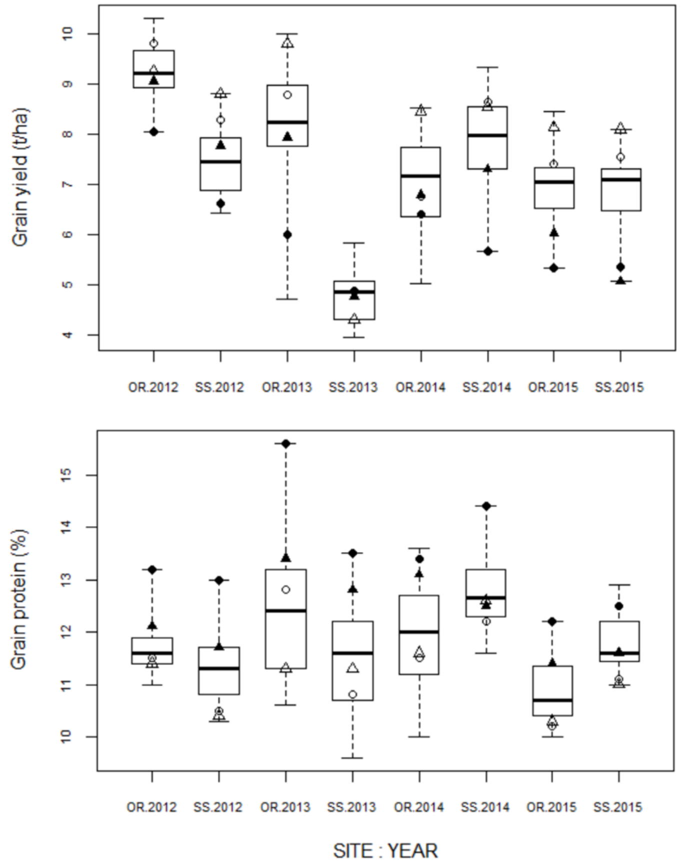

Figure S1. *Grain yield and grain protein for the whole set of 25 cultivars compared at Sassari and Oristano (Sardinia, Italy) from 2012 to 2015. Aureo, full circles; Svevo, full triangles; Iride, empty triangles; Ramirez, empty circles.*

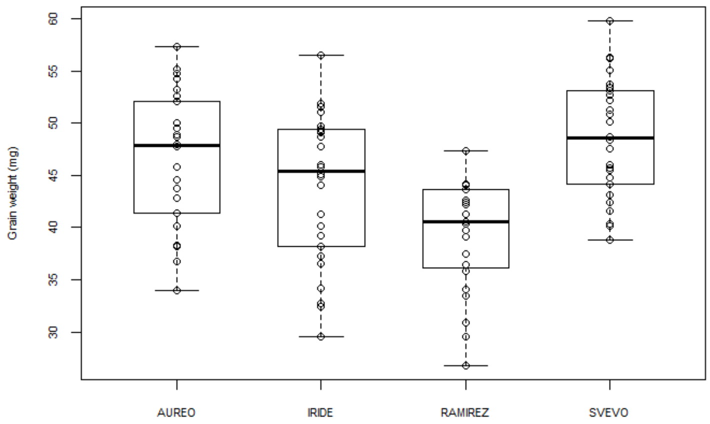

Figure S2. *Mean grain weight recorded for the four cultivars compared across the 27 environmental conditions (9 Nitrogen x Year combination from this experiment plus 18 Site x Year combination from the Italian National Network for durum wheat cultivar comparison)*

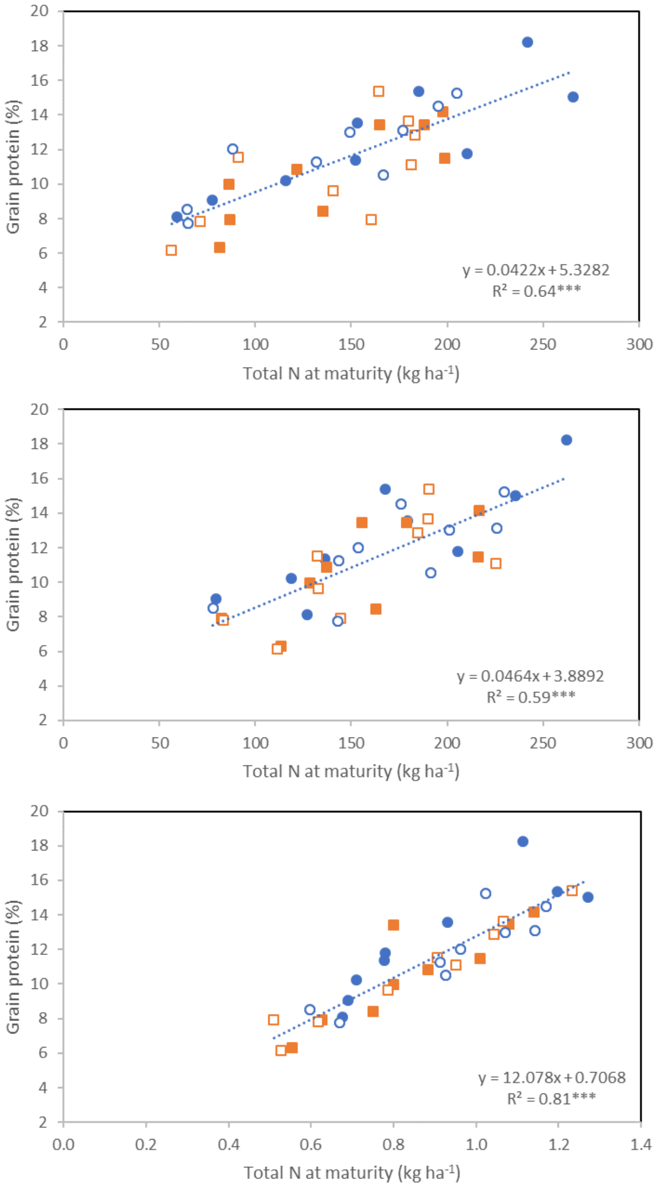

Figure S3. *Relationships between grain protein percentage and total N at anthesis and at maturity, and earNNI. Data are cultivar x year x N treatment means. Aureo, full blue circles; Svevo, empty blue circles; Iride, full orange squares; Ramirez, empty orange squares. ***, significant at P ≤ 0.001.*

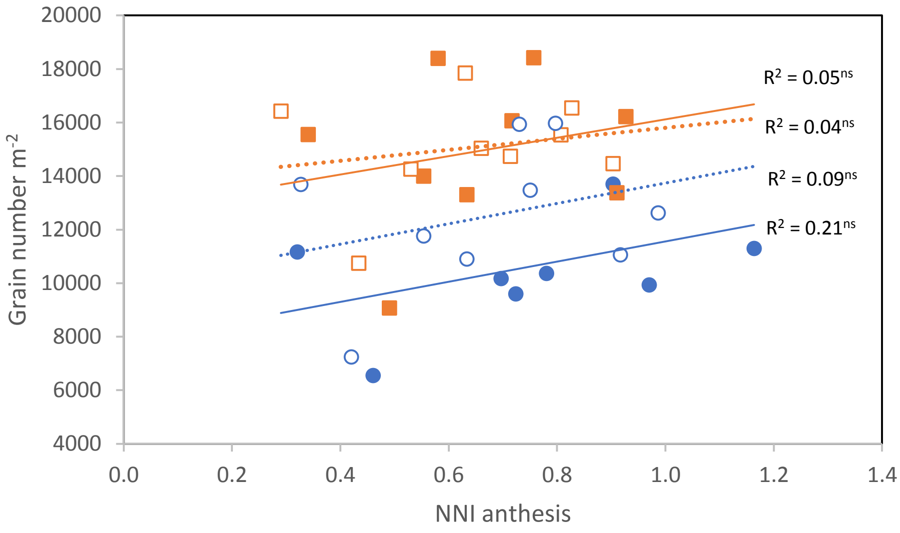

Figure S4*. Relationships between grain number m^{-2} and NNI at anthesis. Data are cultivar x year x N treatment means. Aureo, full blue circles; Svevo, empty blue circles; Iride, full orange squares; Ramirez, empty orange squares. ns, not significant.*