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Nitrogen Metabolism at Tillering Stage Differently Affects the Grain Yield and Grain Protein Content in Two Durum Wheat Cultivars

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Abstract: Soil nitrogen abundance, as well as nitrogen use efficiency (NUE), significantly affect the crop yield and grain protein content (GPC). Depending on the genotype, a negative correlation between the yield and GPC can occur. The aim of the study was to assess the agronomic performance, and to explore physiological pathways for the efficient use of N fertilizer for two durum wheat cultivars, "Aureo" and "Vespucci". After fertilization, the nitrogen content and values of some of the agronomic parameters and yield-related traits increased in both cultivars; nevertheless, a simultaneous rise in both the yield and GPC occurred only in Aureo. The biochemical parameters, analyzed at tillering, confirm the genotypic specificity of nitrogen use. In Vespucci's roots, the nitrogen supply did not affect the nitrate reductase (NR), but greatly increased the amino acids and proteins, suggesting that ammonium is preferentially assimilated. In Aureo, nitrate is in part assimilated by the roots, as suggested by the ammonium increase and NR enhancement. In the leaves of both cultivars, organic nitrogen significantly increased after fertilization; however, the rise in amino acids, as well as in NR activity, was higher in Aureo than in Vespucci. These results indicate that the different nitrogen use, and in particular the diverse NR behavior, at tillering, are in part responsible of the cultivar differences in grain yield and GPC.

Keywords: durum wheat; grain protein content; grain yield; nitrogen metabolism; nitrogen use efficiency

1. Introduction

Nitrogen is an essential element playing a crucial role in plant growth and development, as it is involved in the biosynthesis of several molecules, such as amino acids, proteins, nucleotides, nucleic acids, chlorophyll, vitamins, and hormones [1,2]. Thus, considering its importance as a limiting factor, its abundance in the soil significantly affects both crops' quality and yield [3]. Durum wheat (*Triticum turgidum* var. *durum* Desf.) represents about 5% of the total global wheat production, with Italy being

the major producer, with an average of almost 4.0 MMT in the 2015–2016 cropping season (International Grain Council, https://www.igc.int/en/default.aspx).

The economic value of this crop depends on several agronomic traits, such as grain yield and grain protein content (GPC), the latter being directly related to both the nutritional and technological values of the final products [4,5]. As reported by several authors, both traits are strictly affected by nitrogen metabolism during plant growth [6,7]. Indeed, a stable QTL (Quantitative Trait Locus) for GPC and grain protein deviation (GPD) were found to be co-migrating with nitrogen-related genes [8]. As typical quantitative traits, their expression is regulated by a complex genetic system, and is affected by environmental factors, as well as genotype and management practices [9]. Breeding programs aimed at the constitution of more efficient varieties, and genetic studies on the identification of the key elements involved in nutrient utilization will surely benefit from the recent release of the durum wheat genome assembly, a tool of great importance for studying durum wheat breeding and gene function, as well as QTL analysis for relevant agronomic traits [10]. Currently, to solve issues of soil N shortage and to increase crop yields and GPC, mineral N fertilizers are worldwide extensively used in intensive farming practices [11]. However, depending on the species and cultivar, only 30–50% of the N supplied to the soil is absorbed by the plants, and the remaining part is lost into the environment by leaching, denitrification, and volatilization [12].

To prevent these issues and improve N utilization, some agronomical strategies have been developed, such as late and split nitrogen applications, which improved the GPC [13–15]. The understanding of how plants respond to and use the available N in the soil has attracted considerable interest from the scientific community [16]. Plant nitrogen-use efficiency (NUE) depends on different steps that consider not only N uptake, but also N assimilation and remobilization [1]. In wheat, as well as in many other crops, NUE is a genotype-dependent response [17–19].

To date, few attempts have focused on defining nitrogen fertilizer efficiency in durum wheat [20,21]. N can be absorbed and utilized by plants, from both organic and inorganic sources, but generally, nitrate and ammonium are the most commonly used forms, as they are more available and more easily converted in organic compounds [3]. Thus, NUE is directly linked to nitrate and ammonium assimilatory pathways. Nitrate absorbed from the soil can be stored or assimilated in the roots, or transported via xylem to the shoot. Depending on the nitrate concentration in the soil, environmental conditions, and genotypes, nitrate assimilation can occur in the roots or shoots [22]. In order to be assimilated, nitrate has to be firstly reduced to ammonium, in a two-step reaction. In the first step, occurring in the cytosol, nitrate reductase (NR) reduces nitrate to nitrite; nitrite is then further reduced to ammonium by nitrite reductase (NiR) localized in plastids [23,24].

In cereals, the NR activity has been correlated with the grain yield and GPC, and used as a marker to estimate the N status of the plant [25–28].

Ammonium, taken directly from the soil or derived from nitrate reduction, is assimilated into amino acids through the activity of glutamine synthetase (GS) and glutamate synthase (GOGAT), which work synergistically [29]. GS and GOGAT are highly responsive to N supply [30,31]. GS catalyzes the amidation of the γ-carboxyl group of glutamate to form glutamine. GOGAT transfers ammonium from glutamine to 2-oxoglutarate to form two glutamate molecules [32]. In plants, two different isoforms of GOGAT exist, namely: ferredoxin (Fd)-dependent and NADH-dependent, which are active in photosynthesizing and non-photosynthesizing cells, respectively [33,34]. Both GOGAT genes have found to be associated with GPC in durum wheat [35,36]. Furthermore, GS has been proposed as a candidate gene for improving NUE by several authors, and its relationship with GPC has also been widely reported [37–41].

The aim of the present work was to study if and how inorganic N fertilization could affect the grain yield and GPC of two durum wheat genotypes, Aureo and Vespucci, which were chosen because they are the genotypes that exhibit an elevated yield and GPC [42,43]. Also, in the present study, we wanted to assess the N fertilization effects on the above-mentioned agronomic traits for these two durum wheat genotypes, and to explore whether the different improvements in the yield and GPC

observed in the two genotypes after N fertilization could depend on differences in the N metabolism in the roots and leaves at the tillering stage.

2. Materials and Methods

2.1. Plant Material and Field Experiment Design

Two durum wheat cultivars, Aureo and Vespucci, parents of a segregant recombinant inbred lines (RIL) population, were chosen from a wider collection of 39 tetraploid genotypes included in the "National Trials", carried out in South-Italy in the 2014–2015 and 2015–2016 growing seasons, evaluated for both quality and yield related traits [42,43]. Based on the data collected in these trials, the aforementioned two cultivars were chosen, showing a similar value of grain yield components and GPC, in order to study whether inorganic N fertilization could affect the two traits.

The experimentation was carried out in the 2016–2017 growing season in open air, in containers of 0.72 m Ø and 0.60 m high, filled with 293 kg of clayey–silty granulometry, composed of the following: 58% total sand, 21.5% silt, and 20.5% clay. Details about the particle size, chemical properties, and hydrologic properties of the soil used in the trial are reported in the Table S1.

The characterization of the soil was performed using the official methodologies [44]. Plants were sowed in November, with a sowing rate of 350 seeds m^{-2}. Total rainfall during the growing season was 451 mm. A detailed thermo-pluviometric trend is reported in Supplementary Figure S1. The two genotypes were grown in a complete randomized block design with three replications for both the control and fertilized plants. The plants were supplied with mineral fertilizer and compared with a control test grown without fertilizer. We used 120 U/ha of ammonium nitrate (1:1 ratio) as the mineral fertilizer; 30% of which was supplied during the seedling stage (stage 12 in the Zadoks scale; hereafter indicated as N36), and the remaining 70% during the flowering stage (stage 61 in the Zadoks scale; hereafter indicated as N120). At the tillering stage (stage 24 in the Zadoks scale), the roots and leaves were collected from the control and fertilized plants, frozen in liquid nitrogen, and stored at −80 °C. The sampling, made by pooling five different plants for each container in triplicate, was carried out in the morning, between 09:00 and 11:00.

2.2. Agronomic, Productive, and Qualitative Parameters

The chlorophyll content per leaf area was measured with a Minolta SPAD 502 Chlorophyll Meter (Minolta, Osaka, Japan) on a flag leaf at the booting and flowering stages. The pants were harvested at maturity, and both the agronomic- and yield-related traits were evaluated. Specifically, the aerial biomass, plant height, number of culms, and number of spikes were retrieved, along with a thousand kernels' weight, hectolitre weight, grain weight, and grain yield data [45].

The grain protein content (GPC) was assessed on 3 g of whole meal flour using a dual beam near infrared reflectance spectrophotometer (Zeutec Spectra Alyzer Premium, ZeutecBüchi, Rendsburg, Germany).

2.3. Intracellular Inorganic and Organic Nitrogen Pools

To determine the inorganic N and free amino acids, the plant roots and leaves collected in the tillering stage were kept frozen in liquid nitrogen, and were ground in a mortar with 1:10 (*w/v*) H_2Od. Polyvinylpyrrolidone (PVP; 0.01g mL^{-1}) was added to the crude extracts, which were incubated for 1 h at room temperature. After the incubation, the extracts were centrifuged at 16,000 × *g* for 15 min at 4 °C, and supernatants collected for the analyses.

For the ammonium determination, 50 µL of supernatant was added to 550 µL H_2Od and 25 µL Nessler's reagent (Sigma-Aldrich®, Saint Louis, Missouri, USA). The samples were incubated for 15 min at room temperature, and the ammonium concentration was measured by UV-VIS spectrophotometry at A420 nm. The results were expressed as µg g^{-1} FW.

The nitrate and nitrite contents were measured with the Griess method according to Verdon et al. [46]. In this method, the nitrate was reduced to nitrite by nitrate reductase with a low concentration of NADPH, in order to avoid the interference of $NADP^+$ with the Griess reaction. This reaction was coupled with a glucose-6-phosphate dehydrogenase reaction to ensure a continued reduction of NADPH.

The content of the free amino acids was determined with the ninhydrin method, as described by Magné and Larher [47]. Briefly, 200 µL of leaf or root extracts were incubated with 100 µL of 0.2 M sodium citrate buffer (pH 4.6), and 200 µL of 1% ninhydrin and 0.03% ascorbate solution. After 15 min of incubation at 100 °C, the reaction was stopped in ice, and 600 µL of 60% ethanol was added. The absorbance was read at 570 nm in a Beckmann DU 6400 spectrophotometers (Beckmann Coulter, Brea, California, USA). The amino acid content was expressed as µmoles g^{-1} FW.

The soluble proteins were assayed according to Bradford [48], using bovine albumin as a standard.

For the determination of the photosynthetic pigments, the leaves were frozen in liquid nitrogen and ground in a mortar with 1:30 (*w/v*) 80% acetone. The crude extracts were centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatants were used to determine the absorbance at 663.2, 648.8, and 470 nm, by visible spectrophotometry. The content of the total chlorophylls was calculated as described by Zhang and Kirkham [49].

2.4. Enzymatic Activities Assay

Roots and leaves, kept in liquid N, were ground in a mortar with 1:5 (*w/v*) extraction buffer (50 mM potassium phosphate buffer pH 7,5; 1 mM EDTA (Ethylenediamine tetraacetic acid); 1 mM DTT (Dithiothreitol); 0,1 mM PMSF (phenylmethylsulfonyl fluoride); 0.1 g/L PVP (Polyvinylpyrrolidone)). The crude extracts were centrifuged at 20,000× *g* for 15 min at 4 °C, and the supernatants used for the determination of the enzymatic activities.

The maximal activity of NR (EC 1.7.1.1) was substantially detected, as described by Gibon et al. [50]. Briefly, one volume of extracts was incubated with five volumes of buffer (50 mM Hepes/KOH, pH 7.5, 0.04% (*v/v*) Triton X-100, 2 mM EDTA, 10 µM Na_2MoO_4, 20 µM flavin adenine dinucleotide, 0.5 mM DTT, 20 µM leupeptin, 20 mM potassium nitrate). The reaction was started by the addition of 0.6 mM of NADH. After 15 min, 300 µL aliquots were kept, and the reaction was stopped by adding 25 µL of 0.6 mM zinc acetate. Finally, 300 µL 1% (*w/v*) sulfanilamide in 3 N HCl and 300 µL 0.02% (*w/v*) N(1-naphtyl)ethylendiamine dihydrochloride in 2.5% (*v/v*) H_3PO_4 were added. After 20 min, the absorbance was read at 540 nm.

The NiR (EC 1.7.2.2) activity was detected, as described by Takahashi et al. [51]. The GS (EC 6.3.1.2) activity was detected, as described by Nigro et al. [39]. The NADH-GOGAT (EC. 1.4.1.14) and Fd-GOGAT (EC 1.4.7.1) activities were detected, as described by Esposito et al. [52].

2.5. Statistical Analysis

The data were analyzed as a randomized design with three biological and five technical replications, and expressed as means ± SE. Statistical analysis was carried out using Sigma Plot software 12.0 (Systat Software, Inc., San Jose, CA, USA). One-way analysis of variance (ANOVA) and Tukey's comparison test were used to calculate the difference between the two genotypes and the treatments. Differences were considered statistically significant at a p-value of < 0.05.

3. Results

3.1. Effects of Nitrogen Fertilization on Agronomic, Yield, and Qualitative Traits in Two Durum Wheat Cultivars

The nutritional state of wheat plants was indirectly determined during the booting and flowering stages, through the analysis of the total chlorophyll content obtained by the Soil Plant Analysis Development (SPAD) chlorophyll meter measurements. The Aureo and Vespucci cultivars, grown

without fertilization (N0), did not show significant a difference in SPAD values in both of the growth stages; N fertilization (N120) caused a significant increase in the SPAD values, which, however, did not differ among the two cultivars in both the booting and flowering stages (Figure 1).

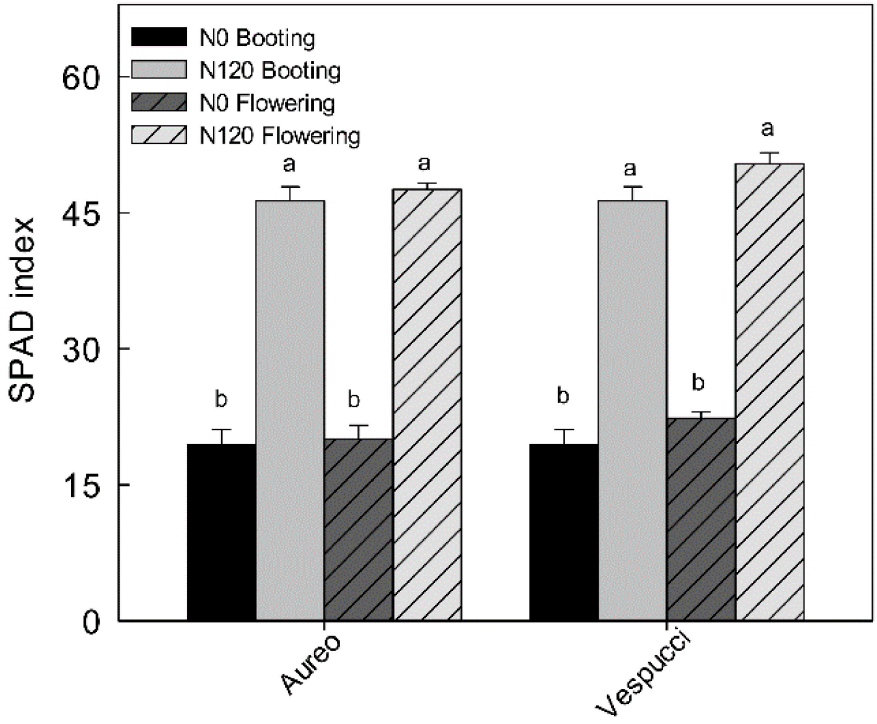

Figure 1. Nitrogen fertilization effect on Soil Plant Analysis Development (SPAD) index during the booting and flowering stages in two durum wheat cultivars. N0 = control condition without nitrogen supply; N120 = 120 U/ha of nitrogen fertilization as ammonium nitrate. Data are the means ± SE of the traits. Data are the means ± SE of five experiments; different letters indicate significant differences (one-way ANOVA test; $p < 0.05$).

To estimate the possible changes in plant growth due to N treatment, yield-related agronomic traits, such as the aerial biomass, plant height, and number of culms and spikes, were measured at the harvesting time. These traits did not vary significantly among the two genotypes grown under the control conditions (N0). N fertilization (N120) caused an increase in aerial biomass and in the number of culms and spikes of both cultivars; conversely, the plant height in the fertilized plants increased significantly only in Vespucci (Table 1).

Table 1. Influence of nitrogen fertilization on agronomic traits at the harvesting stage. N0 = control condition without nitrogen supply; N120 = 120U/ha of nitrogen fertilization. Data are the means of five independent measurements ± SE. Different letters represent values significantly different ($p \leq 0.05$; Tukey's test).

Genotypes and Treatment		Aerial Biomass (g/m^2)	Plant Height (cm)	Number of Culms ($n°/m^2$)	Number of Spikes ($n°/m^2$)
Aureo	N0	$409^{b} \pm 58$	$68.3^{b} \pm 1.7$	$318.0^{b} \pm 10$	$292.4^{b} \pm 1.8$
	N120	$1473^{a} \pm 49$	$80.7^{a,b} \pm 1.7$	$500.8^{a} \pm 3.8$	$465.2^{a} \pm 3.4$
Vespucci	N0	$337^{b} \pm 43$	$64.3^{b} \pm 3.4$	$316.7^{b} \pm 10.3$	$288.6^{b} \pm 2.1$
	N120	$1678^{a} \pm 44$	$90.0^{a} \pm 2.9$	$482.6^{a} \pm 14.4$	$454.5^{a} \pm 10.9$

The effects of N fertilization on the thousand kernel weight (TKW), grain yield, and hectolitre weight for the two cultivars are compiled in Table 2. In the absence of fertilization, only the thousand kernels' weight differed among the two cultivars, being higher in Vespucci than in Aureo. Under N fertilization, the three analyzed parameters increased in both of the cultivars, even if the increase in the thousand kernels' weight, and especially in the grain yield, was significantly higher in Vespucci than in Aureo. These data suggest that, at least for the yield, Vespucci was more responsive than Aureo to N fertilization. On the contrary, GPC, taken into consideration as a qualitative trait, did not differ in the two cultivars grown under the control conditions (N0), and increased significantly under N fertilization only in Aureo (Table 2).

Table 2. Nitrogen fertilization effects on the yield and quality traits at the harvesting stage. N0 = control condition without nitrogen supply; N120 = 120U/ha of nitrogen fertilization. Data are the means of five independent measurements ± SE. Different letters represent values significantly different ($p \leq 0.05$; Tukey's test).

Cultivars and Treatment		Thousand Kernels Weight (g)	Hectolitre Weight (g/hl)	Grain Yield (g/m^2)	Grain Protein Content (%)
Aureo	N0	$43.0^{c} \pm 1.1$	$78.3^{b} \pm 1.3$	$152^{c} \pm 26$	$11.4^{b} \pm 0.1$
	N120	$45.6^{b} \pm 1.3$	$81.3^{a,b} \pm 2.3$	$642^{b} \pm 38$	$14.2^{a} \pm 1.3$
Vespucci	N0	$45.7^{b} \pm 1.7$	$79.3^{b} \pm 2.3$	$144^{c} \pm 21$	$10.8^{b} \pm 0.2$
	N120	$51.3^{a} \pm 1.9$	$83.3^{a} \pm 2.3$	$773^{a} \pm 38$	$12.1^{b} \pm 0.3$

3.2. Nitrogen Metabolism in Two Wheat Cultivars during Tillering Stage

Considering the positive effects of N fertilization on some of the parameters of productivity and/or GPC in Vespucci and Aureo, it was verified whether the different improvement in the two cultivars could depend on different changes in the N metabolism of roots and leaves in the early phases of plant growth (i.e., during the tillering stage).

The intracellular inorganic and organic N levels in the roots of the two cultivars, grown with or without N fertilization, were determined (Figure 2).

The content of ammonium ions was significantly different in the two cultivars grown without fertilization, being higher in Vespucci than in Aureo. Notably, the two cultivars responded differently to N fertilization, as follows: the ammonium ion increased and decreased in the Aureo and Vespucci roots, respectively (Figure 2A). On the other hand, Aureo, without fertilization, showed a higher nitrate content than Vespucci; in both cultivars subjected to N fertilization, a significant increase in this ion occurred (Figure 2B). The intracellular content of nitrites was not detectable in either the control or under N fertilization in both of the cultivars (data not shown).

The level of free amino acids and soluble proteins did not significantly differ among the two cultivars grown without fertilization (Figure 2C,D). Under N treatment, the free amino acids significantly increased in both cultivars, even if the increment was greater in Vespucci than in Aureo (Figure 2C). Similarly, the N fertilization caused an increase of soluble proteins only in the Vespucci cultivar (Figure 2D).

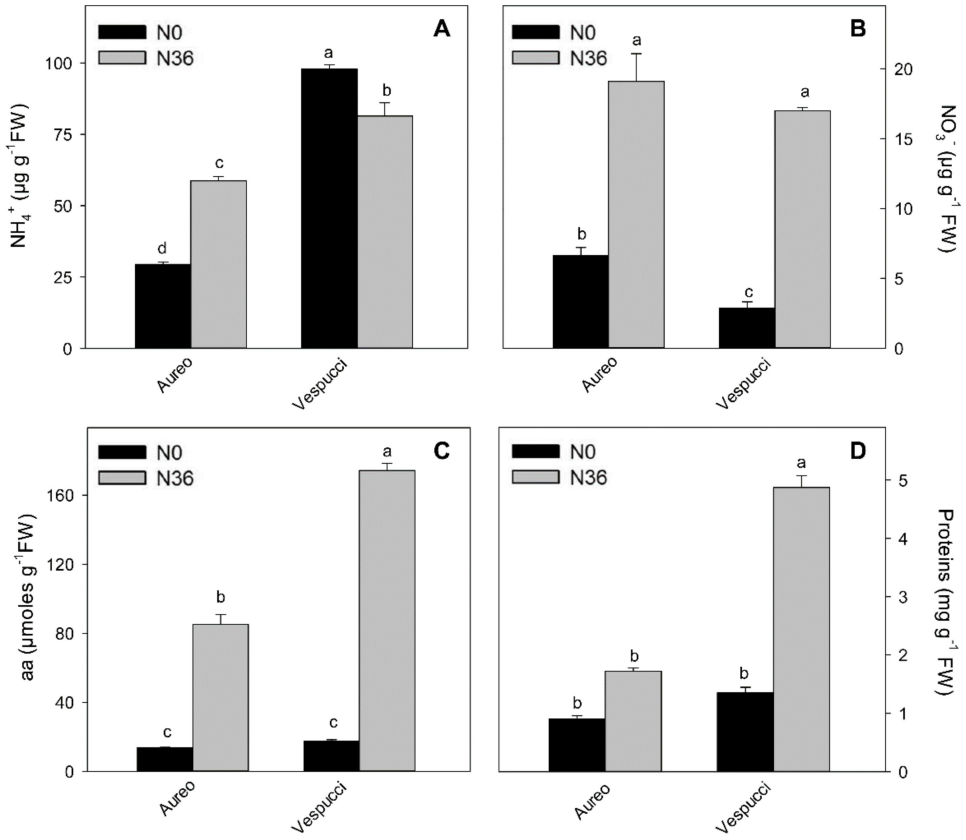

Figure 2. Nitrogen fertilization differently affects the nitrogen content in the roots of two wheat cultivars at the tillering stage. Intracellular content of ammonium (**A**); nitrate (**B**); amino acids (**C**); soluble proteins (**D**). N0 = control condition without nitrogen supply; N36 = 36 U/ha of nitrogen fertilization as ammonium nitrate. Data are the means ± SE of five experiments; different letters indicate significant differences (one-way ANOVA test; $p < 0.05$).

The activity of the enzymes involved in the N assimilation was also measured in the roots of both cultivars, grown with and without N fertilization (Figure 3).

The NR and NiR activities did not show significant differences among the two cultivars grown in the control conditions (Figure 3A,B). Under N fertilization, an increase in NR activity occurred only in Aureo (Figure 3A). In parallel with the ammonium content (Figure 2A), the GS activity was greater in the Vespucci roots than in the Aureo ones; however, fertilization stimulated the GS activity in both of the genotypes (Figure 3C). The NADH-GOGAT activity did not show a significant difference among the two durum cultivars without fertilization, and an increase occurred as a consequence of N supplementation (Figure 3D).

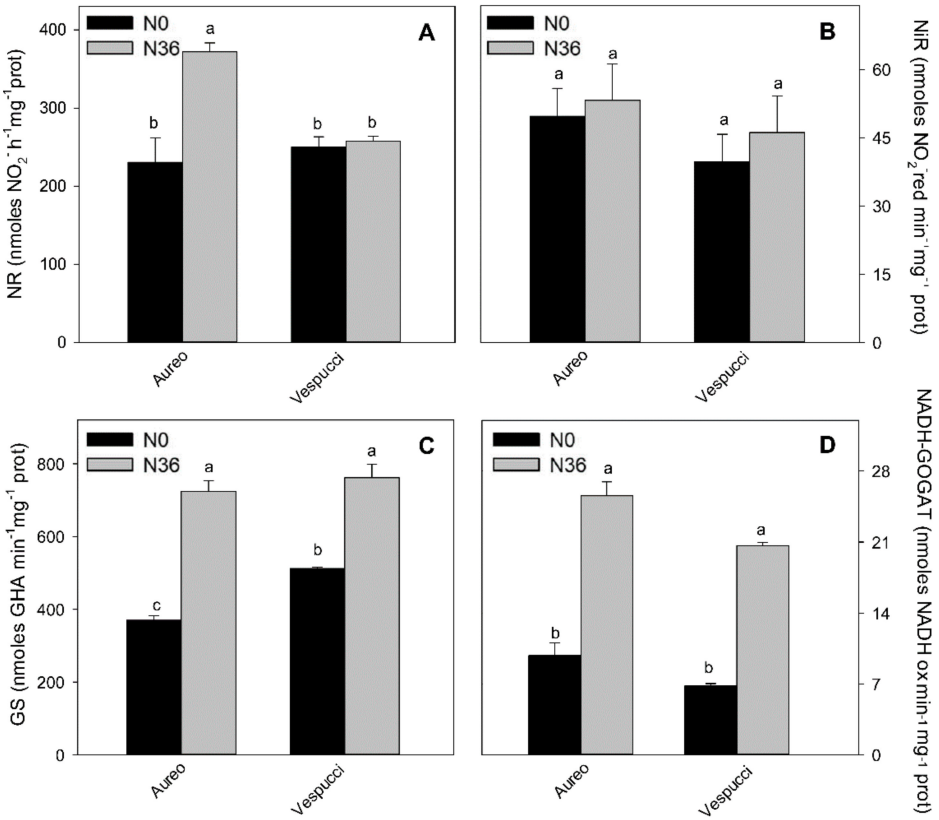

Figure 3. Influence of nitrogen fertilization on the enzymes of nitrogen metabolism in the roots of two wheat cultivars at the tillering stage. Specific activities of (**A**) nitrate reductase (NR), (**B**) nitrite reductase (NiR), (**C**) glutamine synthetase (GS), and (**D**) glutamate synthase (NADH-GOGAT). N0 = control condition without nitrogen supply; N36 = 36 U/ha of nitrogen fertilization as ammonium nitrate. Data are the means ± SE of five experiments; different letters indicate significant differences (one-way ANOVA test; $p < 0.05$).

The N content and metabolism has also been studied in the leaves of the two cultivars at the tillering stage (Figures 4 and 5). As observed in the roots, the intracellular ammonium content was higher in the Vespucci leaves than in Aureo's; the N supplement sharply reduced the content of this ion in both cultivars (Figure 4A). The nitrate content was comparable in the two cultivars grown without fertilization, and it increased approximately 100 times under ammonium nitrate treatment (Figure 4B). The nitrite content in the leaves was very low, and was not affected under fertilization in the two genotypes (data not shown). Despite the fact that in the control conditions, the amino acid pool was higher in Vespucci than in Aureo leaves, under N fertilization, this pool increased more in Aureo than in Vespucci (Figure 4C). Soluble proteins had comparable levels in the two cultivars grown in the absence of fertilization; N supplementation determined a significant increment, which did not show differences among the genotypes (Figure 4D). Chlorophylls, which represent another form of organic N storage, showed the same behavior of soluble proteins, increasing after N fertilization in the same way in both cultivars (Figure 4E,F).

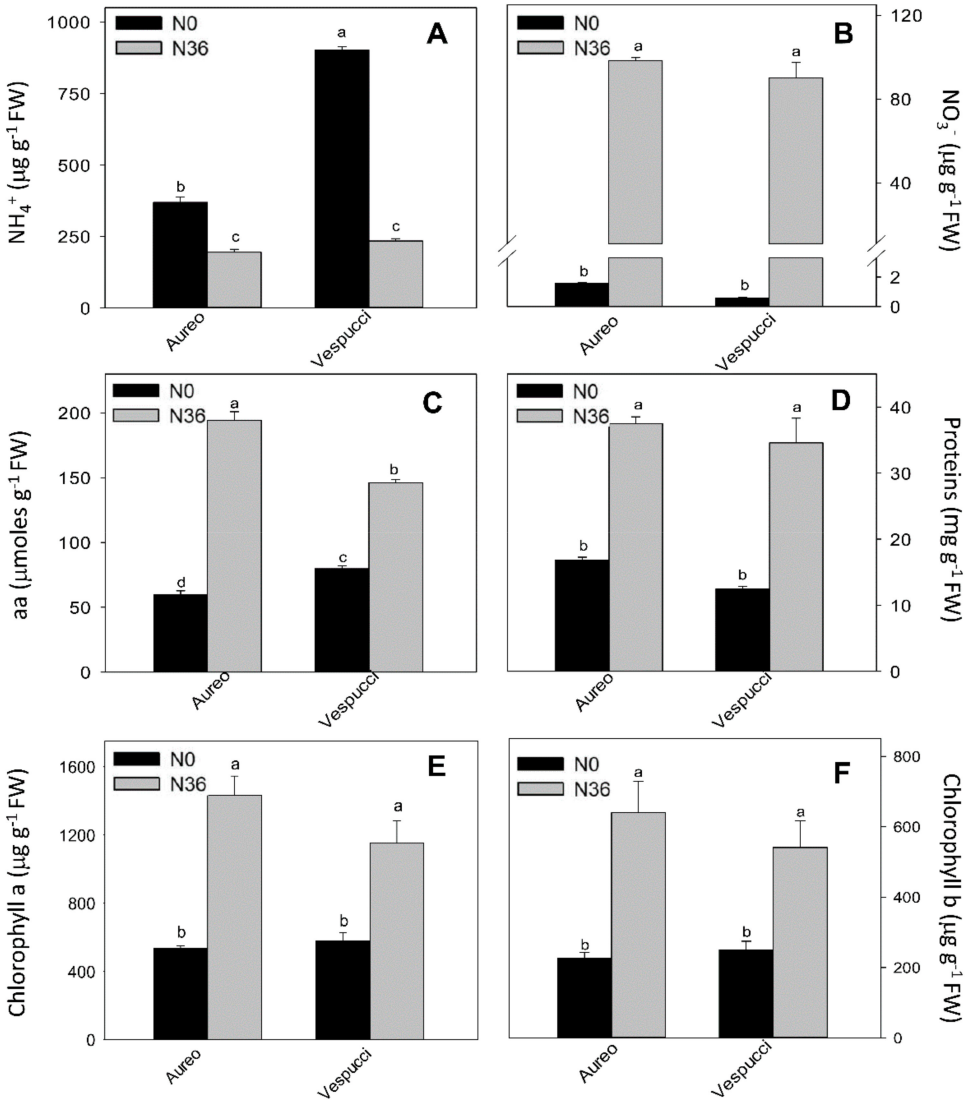

Figure 4. Effects of nitrogen fertilization on the nitrogen content in the leaves of two wheat cultivars at the tillering stage. Intracellular content of ammonium (**A**); nitrate (**B**), amino acids (**C**), soluble proteins, (**D**), Chlorophyll A (**E**), and Chlorophyll B (**F**). N0 = control condition without nitrogen supply; N36 = 36 U/ha of nitrogen fertilization as ammonium nitrate. Data are the means ± SE of five experiments; different letters indicate significant differences (one-way ANOVA test; $p < 0.05$).

The NR activity, which was not statistically different in the Vespucci and Aureo leaves under the control conditions, increased significantly with the N fertilization, reaching values significantly higher in Aureo than in Vespucci (Figure 5A).

The NiR activity was also induced by N, and showed comparable values in both cultivars (Figure 5B). GS and Fd-GOGAT showed a similar activity in the leaves of both cultivars grown without fertilization (Figure 5C,D). When N was supplied, contrarily to what occurred in the roots, the GS activity significantly decreased in both genotypes (Figure 5C), and the Fd-GOGAT did not change in Aureo, and resulted in inhibition in Vespucci (Figure 5D).

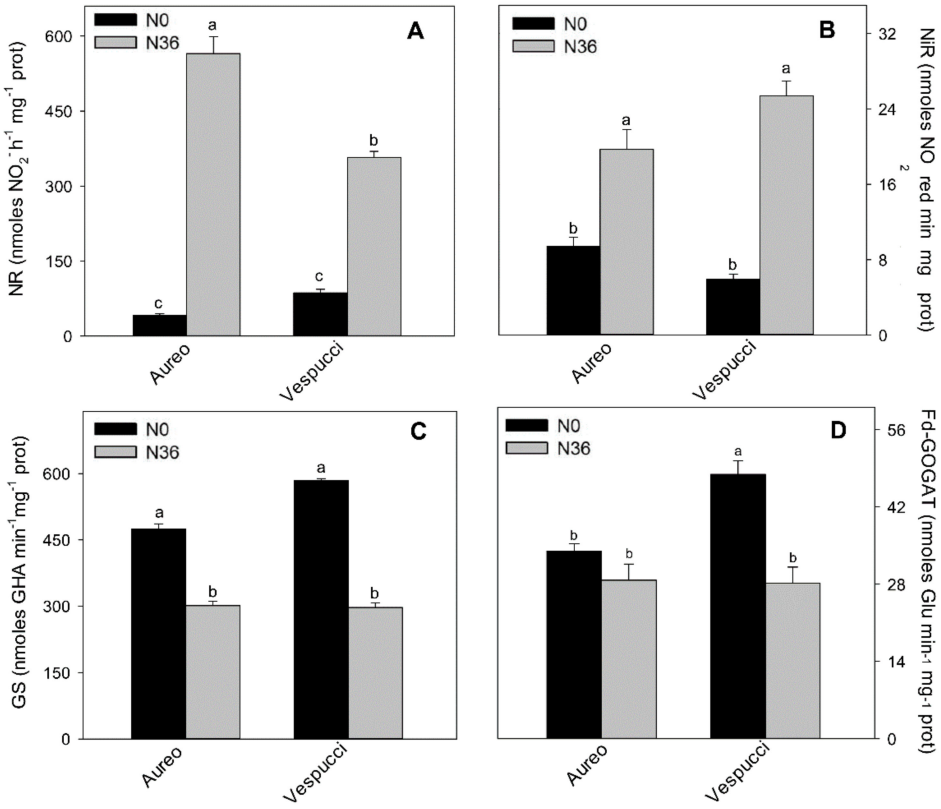

Figure 5. Influence of nitrogen fertilization on enzymes of nitrogen metabolism in the leaves of two wheat cultivars at the tillering stage. Specific activities of (**A**) nitrate reductase (NR), (**B**) nitrite reductase (NiR), (**C**) glutamine synthetase (GS), and (**D**) glutamate synthase (NADH-GOGAT). N0 = control condition without nitrogen supply; N36 = 36 U/ha of nitrogen fertilization as ammonium nitrate. Data are the means ± SE of five experiments; different letters indicate significant differences (one-way ANOVA test; $p < 0.05$).

4. Discussion

N fertilization is one of the strategies more frequently used to increase crop yield [11]. Accordingly, our results show that after fertilization, N is absorbed and assimilated by both of the genotypes, as demonstrated by the increase in SPAD index in both the booting and flowering stages (Figure 1), which can be considered as an indirect measure of the N content of leaves [53–56]. The N availability for plants after fertilization is also deduced by the analyzed agronomic traits, such as the number of culms, number of spikes, and aerial biomass, which significantly increase in both genotypes (Table 1). In accordance with the literature data, the increase in aerial biomass can be due to an increase in the photosynthetic surface, and to a higher ability to produce reserve compounds, which results in a greater productivity, such as the number of grain/m^2 [57–60]. N fertilization increases all of the yield-related traits in both genotypes, but this increment is higher in Vespucci than in Aureo (Table 2). In wheat, N fertilization can also increase the N content of kernels, and consequently GPC [61], but this character shows a negative correlation with yield, whose extent is closely dependent on the genotype [62,63]. In Aureo, a simultaneous increase in both the yield and GPC occurs (Table 2), which highlights that this cultivar behaves as an optimal one, able to combine good yields with a high GPC [64,65]. The concomitant increase of yield and GPC in Aureo could be due to the efficient N translocation to the developing grains, as already suggested in previous papers [66,67], or to the different N uptake efficiency from the soil, probably attributed to its specific root architecture. It is known that wheat piles up most of its N before the booting and flowering stage. A good correlation between plant growth-related aspects during the early stages of the wheat vegetative cycle and final

yield has been largely reported [68,69]. Furthermore, in bread wheat, 60–95% of the N in the grains is derived from the remobilization of N accumulated in the roots and shoots before anthesis [37,70].

The exploitation of the genotype efficiencies in ammonium and nitrate use is of great importance to optimize not only NUE, but also grain quality. Our analyses on N metabolism, carried out during the tillering stage, show a complex metabolic scenario, which could be, in part, responsible of the differences in yield and GPC, occurring in the two wheat genotypes after inorganic N fertilization.

Without N fertilization, Vespucci's roots show a higher amount of intracellular ammonium, as well as of GS activity, than Aureo; after the N supply, a decrease in intracellular ammonium and a significant increase in amino acid pool and soluble proteins occur (Figure 2), suggesting that Vespucci's roots preferentially absorb and assimilate ammonium rather than nitrate. On the other hand, Aureo's roots have a higher nitrate content than Vespucci's; after the N supply, only Aureo's roots show an enhancement of NR activity (Figure 3). This different behavior of the two genotypes confirms that N absorption and assimilation are genotype specific [39,71,72].

In both genotypes, under control conditions, the NiR activity in the roots is significantly higher than the NR, and it does not increase after fertilization (Figure 3), confirming that this enzyme is not limiting. The high NiR activity makes sure that nitrite is quickly reduced to ammonium, to avoid its accumulation, which could be extremely harmful for cells [73]. Consistently, in both genotypes, nitrite is undetectable with or without N fertilization. At the same time, GS and GOGAT also behave similarly in the two cultivar's roots (Figure 3). The induction of these enzymes, observed in the roots grown under a high N availability is consistent with the previously published works, which show that ammonium specifically induces GS and NADH-GOGAT in the roots [2,74]. An increase in GS activity in the roots under ammonium nitrate fertilization, has been previously reported in other four different wheat genotypes [39]. The roots are the major site of primary ammonium assimilation, and GS and NADH-GOGAT activities are induced for ammonium assimilation and detoxification [33,71,75].

In leaves, N metabolism shows some important differences between the two genotypes. As observed in the roots, in the leaves without fertilization, ammonium is higher in Vespucci than in Aureo; in this case, the N supply causes a drastic decrease of this ion in both genotypes (Figure 4). Thus, it is reasonable that, ammonium, being toxic even at a low concentration, is principally assimilated in the roots, and not transported to the leaves [76]. On the contrary, nitrate accumulation in leaves is higher than that observed in the roots (Figure 4), proving, in accordance with the literature, that a substantial portion of nitrate absorbed by the roots can be moved in the aerial part of plants [2]. The high nitrate concentration in the leaves induces a significant increase of NR and NiR activity in both genotypes, thus confirming that nitrate functions as an inducer of both enzymes [77,78].

Despite the inorganic N supplement that determined the increment of both NR and NiR activities and the reduction of ammonium levels, the GS activity was inhibited in both wheat genotypes, and the GOGAT activity does not change in Aureo and decreases in Vespucci (Figure 5). This is not surprising, because an inhibition of these enzymes was already observed previously in different wheat genotypes, with a high yield and GPC, growing with the high ammonium nitrate concentration [39,79]. The inhibition of the GS-GOGAT cycle can depend on the high nitrate concentration in leaves [79]. However, consistently with the high yield, the amino acids, proteins, and chlorophylls, which can give an estimation of total N [1], increase significantly after N fertilization in the leaves of both genotypes. It has been suggested that soluble proteins in vegetative organs make an exclusive pool of N accessible for the growth of vegetative and storage organs; the amplitude of this pool mirrors the plant N status [27,80–83].

The full genetic potential of N use is expected to be evident under non-limiting nitrogen supply [84], wherein Aureo and Vespucci differ significantly in their N metabolism. Indeed, although the amino acid content (Figure 4) and NR activity (Figure 5) after N fertilization rise in the leaves of both genotypes, the increase is significantly higher in Aureo than in Vespucci. These two parameters seem to be correlated, as it has been previously reported that the amino acid pool in the leaves could depend on the NR expression [85]. It has been shown that wheat genotypes with a high NR were able to

mobilize a higher amount of N to the grains than the low NR genotypes [86]. Moreover, two wheat genotypes over-expressing a tobacco NR gene increased by 30% GPC, probably because the improved level of NR in the leaves accelerates nitrate assimilation and facilitate the N flux to the seeds during grain development [28]. Interestingly, also in different genotypes of pearl millet, a significant positive correlation among the NR activity at an early vegetative stage and GPC content of mature grains, has been demonstrated [87]. Consistently, our results support the hypothesis that the high induction of NR activity in Aureo after N fertilization could contribute to the increase in GPC. However, our data do not allow us to exclude that an increase in GPC, observed in Aureo after fertilization, can be also due to a high N mobilization, as well as to the plant's ability to utilize the N available in the soil during the ripening stage [1,88,89].

5. Conclusions

Our results indicate that the two genotypes use the inorganic N exogenously supplied differently. Indeed, Aureo can increase both the yield and GPC after fertilization. The analyses of the N metabolism at the tillering stage in the two genotypes confirm that the N absorption and assimilation are genotype specific. Moreover, the increase in NR activity in the roots and leaves after N fertilization is significantly higher in Aureo than in Vespucci. These data could be taken into consideration in future works in order to check if the increase in NR activity under inorganic N fertilization could be used to choose the wheat genotypes able to increment both yield and GPC.

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-2818/11/10/186/s1: Figure S1: Annual rainfall and average temperature data for the cropping season of 2016–2017 detected in the field trial of the University of Bari, Italy. Table S1: Particle size distribution, and chemical and hydrologic properties of the soil used in the trials.

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