Differential responses of grain yield, grain protein, and their associated traits to nitrogen supply in soft red winter wheat

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Abstract

Increased application of nitrogen fertilizers has significantly raised grain yield and protein concentration in wheat. However, only 30–50% of applied fertilizer nitrogen are usually utilized by the plant. In this study, four soft red winter wheat genotypes (*Triticum aestivum* L., IL07-4415, MD05W10208-11-8, OH06-150-57 and Sisson) were grown under three different nitrogen regimes (high, medium, and low) in a greenhouse, and grain yield, grain protein concentration, nitrogen use efficiency (NUE) and their associated traits were evaluated. Among the four genotypes, a high-yielding cultivar, Sisson, exhibited superior performance in terms of grain weight plant^{-1} and NUE for yield (NUEY) at low nitrogen due to maintained grain number spike^{-1} and harvest index. Significant yield losses due to nitrogen limitation were attributable to reduced spike number plant^{-1} and grain number spike^{-1} in the other genotypes. Interestingly, a linear relationship between NUEY and NUE for grain protein (NUEP) was detected at high (R^2 = 0.67) and low (R^2 = 0.42) nitrogen; both of these traits were positively correlated with grain number spike^{-1}, 1000-seed weight, and harvest index under nitrogen-limited conditions (R^2 = 0.35–0.48). These results suggest that simultaneous improvement of NUEY and NUEP could be achieved through the selection of the three yield components (grain number spike^{-1}, 1000-seed weight, and harvest index) at low nitrogen.

Key words: carbohydrates / nitrogen deficiency / nitrogen use efficiency / *Triticum aestivum*

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1 Introduction

Nitrogen (N) is globally the highest consumed mineral macronutrient in today's high-yield agricultural production systems, representing a significant cost for growers. The current usage of N fertilizers is nine-fold greater than that in the 1960's, and application of N is predicted to increase by 40–50% over the next 40 years (*Sutton* et. al., 2013). An increased input of N fertilizers has been indispensable for continuous increases in crop productivity. However, for most cereal species only 30–50% of applied N are actually taken up by the plant in the same season (*Dobermann* and *Cassman*, 2002; *Kitchen* et al., 2008). Nitrogen not absorbed by plants can be released to atmosphere and water bodies, resulting in greenhouse gas emission and eutrophication of freshwater and marine ecosystems (*Vitousek* et al., 1997; *Mueller* et al., 2012; *Zhang* et al., 2015). Optimization of the rate, timing, and placement of N fertilizers and usage of slow and controlled-release fertilizers would be effective approaches for the reduction of ammonium emission, denitrification, N leaching, and runoff from agricultural systems. In addition to these management-based solutions, genetic improvement of nitrogen use efficiency (NUE) in crops must be of necessity to address the complex environmental issue.

Wheat (*Triticum aestivum* L.) is the most widely grown crop in the world. In 2014, the total area harvested was 222 million ha of wheat, as compared with 183 million ha of maize, and 163 million ha of rice (*FAO*, 2014). Protein concentration is a critical factor determining grain quality and marketability in wheat. To achieve the target concentration of protein in grains, a high level of N fertilizers has been supplied in wheat production (*Shewry*, 2009; *Henry* et al., 2016). For these reasons, of the total N produced globally, 18% are applied in wheat cultivation; the largest amount of N supply in crop production (maize 16% and rice 16%; *Ladha* et. al., 2016). Based on the scale of cultivation and the high requirement of N fertilizers, genetic improvement of NUE in wheat can largely contribute to the suppression of water and air pollution caused by excessive N input as well as to the reduction in production costs.

Soft red winter wheat is widely grown over the eastern third of North America. It is generally higher-yielding with lower grain protein concentration than hard wheat (*Thomason* et al., 2009). As in hard wheat, grain protein concentration is an important parameter to evaluate the end-use quality of soft red winter wheat (*Souza* et al., 2011; 2012). Nitrogen use efficiency for yield (NUEY) and protein (NUEP) are defined as grain dry weight or total grain N divided by fertilizer N supplied

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to the crop, respectively (*Moll* et al., 1982; *Van Sanford* and *MacKown*, 1987). It was previously reported that significant genotypic variations for NUEY and NUEP exist in soft red winter wheat (*Van Sanford* and *MacKown*, 1987; *Pavuluri* et al., 2014).

Identification of morphological and physiological components that are positively and negatively associated with NUEY and NUEP is a necessary step for dissecting the genetic basis of these complex traits. In this project, we evaluated the effect of N supply on grain yield, grain protein concentration, and their associated traits under three N regimes in four soft red winter wheat genotypes with diverse NUEY and NUEP. This study allowed us to identify key traits which are strongly correlated with NUEY and NUEP, useful diagnostic traits for selection of high NUE accessions in soft red winter wheat.

2 Material and methods

2.1 Plant materials and growth condition

Four soft red winter wheat accessions, *Triticum aestivum* L. IL07-4415 (IL), MD05W10208-11-8 (MD), OH06-150-57 (OH), and Sisson were analyzed in this study. These accessions were selected based on the genetic diversity survey for NUE within a panel of 281 soft red winter wheat genotypes regionally developed in the eastern U.S. (*Pavuluri* et al., 2014). This study was conducted from October 8, 2014, to May 7, 2015, in a greenhouse located in Blacksburg, Virginia, USA (37°12'N, 80°24'W). Seeds were sterilized in 2.5% (v/v) sodium hypochlorite and 0.1% (v/v) Tween-20 for 5 min and thoroughly rinsed with deionized water. Sterilized seeds were placed on wet paper towels for 4 d at 25°C in the light (50 μmol m^{-2} s^{-1}). Germinated seedlings were then transplanted into plastic pots [5.7 cm (L) × 3.8 cm (W) × 5.4 cm (H); 1 plant pot^{-1}] containing Metro Mix 360 potting soil (Sun Gro Horticulture, Vancouver, Canada) and grown in a greenhouse (natural light, 22°C day/13°C night) until the 3-leaf stage. Subsequently, seedlings were exposed to vernalization treatment at 7°C under 8 h light (80 μmol m^{-2} s^{-1}) / 16 h dark cycles for 52 d. Following vernalization, each plant was grown in a pot (2.4 L) containing a mixture of 50% (v/v) Metro Mix 360 and 50% (v/v) sand under greenhouse conditions (natural light; 18°C day/7°C night). When plants had generated five tillers, the light period and temperature were changed to 16 h day (22°C) / 8 h night (13°C) using greenhouse heating and supplemental light (1000 W metal halide × 4) systems.

A half-strength of Hoagland's minus N solution (50 mL $plant^{-1}$) was supplied twice a week to all plants until grain filling was complete. As an N source, ammonium nitrate was applied with 1/2 Hoagland's solution until the 5-tiller stage (11.4 mg N $plant^{-1}$). After that, three rates of N (high N, 11.4 mg; medium N, 3.8 mg; low N, 1.14 mg) were supplied with 1/2 Hoagland's solution twice a week till the booting stage. After this stage, the amount of N supplied was doubled for all treatments (high N, 22.8 mg; medium N, 7.6 mg; low N, 2.2 mg). The total amount of N supplied for each plant was 423 mg for high N, 225 mg for medium N, and 155 mg for low N, respectively.

240 plants (20 plants × 3 nitrogen treatments × 4 genotypes) were set up in a completely randomized design for this study. Yield components were measured for 14 of the plants, while the remaining 6 plants (2 plants per biological replicate) were randomly selected for flag leaf analysis. At anthesis, flag leaf blades were harvested at midday, immediately frozen in liquid nitrogen, and stored in –80°C until use.

2.2 Yield component analysis

Spikes and above-ground tissue were harvested from each plant at maturity and dried at 65°C for 3 d. Yield components measured include: (1) grain weight $plant^{-1}$, (2) spike number $plant^{-1}$, (3) grain number $spike^{-1}$, (4) 1000-seed weight, (5) spike length, and (6) vegetative biomass weight $plant^{-1}$. Harvest index was calculated as the ratio of grain dry matter to total above-ground dry matter.

2.3 Protein analysis

Protein concentration in grains was estimated with an XDS Rapid Content Analyzer (Foss, Eden Prairie, MN, USA). This method has been previously validated in soft red winter wheat using combustion N analysis (FPS 528; Leco Corporation, St. Joseph, MI, USA) (*Pavuluri* et al., 2014). Protein in flag leaves was quantified using the method of *Tamang* et al. (2014).

2.4 Nitrate, ammonium, and total amino acid assays

Frozen tissues (75 mg) were homogenized in 450 μL of 0.83 M perchloric acid on ice. After centrifugation at 21,000 *g* at 4°C for 20 min, 300 μL of the supernatant were neutralized with 75 μL of 1 M bicin (pH 8.3) and 70 μL of 4 M KOH. The resulting salt was removed by centrifugation at 21,000 *g* at 4°C for 20 min, and the supernatant was used for nitrate and ammonium assays as described in *Alpuerto* et al. (2016). For total amino acids, 80 μL of the neutralized extract were mixed with 20 μL of 3 M MgO and left in an opened 1.5 mL tube at room temperature for 16 h. Following overnight incubation, 80 μL of the solution were mixed with 50 μL of 0.2 mM sodium cyanide resolved in 8 M sodium acetate and 50 μL of 168 mM ninhydrin resolved in 100% 2-methoxyethanol. The mixture was heated at 100°C for 15 min and 1 mL of 50% isopropanol was immediately added to the solution. After cooling, the absorbance of each reaction was measured at 570 nm with a spectrophotometer. Glycine was used as the standard.

2.5 Carbohydrate assays

Total soluble sugars and starch were quantified as described by *Fukao* et al. (2006).

2.6 Chlorophyll measurement

Frozen tissue (50 mg) was homogenized in 3 mL of 100% methanol on ice. After centrifugation at 21,000 *g* at 4°C for 20 min, the absorbance of the supernatant was quantified at

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652.0 and 665.2 nm with a spectrophotometer. Chlorophyll *a* and *b* concentrations were calculated following the method of *Porra* (2002).

2.7 Statistical analyses

Statistical analyses were performed using JMP Pro (ver. 11.0.0) (SAS Institute). Two-way ANOVA with Tukey's honest significant (HSD) test was carried out for comparison of means among accessions and N regimes. Correlation coefficient analysis was conducted to determine R^2 and *p*-value for each comparison. NUEY and NUEP were calculated as total grain dry weight (g plant^{-1}) and total grain N (g plant^{-1}) divided by fertilizer N supplied to the plant (g plant^{-1}), respectively (*Moll* et al., 1982; *Van Sanford* and *MacKown*, 1987).

3 Results

3.1 Effect of N supply on yield components and grain protein

Two-way ANOVA revealed that all of the surveyed components associated with grain yield and NUE were significantly affected by genotypes and N rates (Tab. 1). However, the effect of the genotype × N treatment interaction was significant only on grain weight plant^{-1}, NUEY, vegetative biomass, and grain protein concentration. Grain weight plant^{-1} (equivalent to grain yield) was similar among the four genotypes at high N input, but was reduced differentially in response to lower N supply. Sisson exhibited greater grain weight plant^{-1} at low N. By contrast, grain weight plant^{-1} of MD was lowest at medium and low N due to greater yield reduction than other accessions. Because NUEY is determined by grain weight plant^{-1} divided by the amount of fertilizer N supplied to the plant, genotypic and N effects on NUEY and grain weight plant^{-1} were identical (the results of statistical analysis are identical for these two traits). Grain weight plant^{-1} is the product of spike number plant^{-1}, grain number spike^{-1}, and grain weight. Among them, spike number plant^{-1} was reduced under medium and low N input in MD, Sisson, and OH, whereas this trait was not significantly altered in IL. On the other hand, low N input reduced grain number spike^{-1} in IL, MD and OH, but it did not affect this trait in Sisson. Unlike spike number plant^{-1} and grain number spike^{-1}, the 1000-seed weight was not altered in response to N supply in the four genotypes. Lower N application reduced spike length only in Sisson and OH.

Sisson exhibited the greatest harvest index at medium and low N. In the other three genotypes, the harvest index was significantly lower at medium N as compared to high N. Yet, no difference was detected under high and medium N regimes in Sisson. Vegetative biomass was reduced in response to low N supply in all genotypes except for IL. Reduced N application decreased the level of grain protein, with the diverse magnitude of reduction among the four genotypes. Sisson and IL, which showed relatively high grain weight plant^{-1} at low N, were more sensitive to N limitation than the other two genotypes in terms of grain protein reduction. The inverse relationship was observed in MD. A reduction in N supply from high N to medium N significantly reduced NUEP in all accessions. However, a further reduction in N application rate affected NUEP only in Sisson.

3.2 Effect of N supply on correlations among yield components and grain protein concentration

Correlation analysis of yield components, grain protein concentration, and NUE was performed for each N regime separately (Tab. 2). Correlation values between grain weight plant^{-1} and other traits were identical to those between NUEY and other traits because NUEY is determined by grain weight plant^{-1} divided by the amount of N supplied to the plant, but the N rate is not taken into account to calculate correlation coefficient at each N input. Grain weight plant^{-1} and NUEY were positively correlated with 1000-seed weight and harvest index at all N regimes. Grain protein concentration was inversely associated with 1000-seed weight at the three N levels. NUEP was positively related to harvest index regardless of N regimes. In addition, grain weight plant^{-1} and NUEY were positively associated with NUEP at high and low N.

3.3 Effect of N supply on accumulation of N and C compounds in grains

It is anticipated that N supply changes the composition and abundance of N and C compounds in grains. To evaluate this, the concentrations of protein, total amino acids, ammonium, nitrate, starch, and total soluble sugars were quantified in grains of three plants that were randomly selected from 14 plants used for yield component analysis (Tab. 3). Two-way ANOVA indicated that all components except for starch were significantly affected by genotypes. N supply did not influence only the abundance of soluble sugars. The interaction between genotypes and N treatment was significant in total amino acid, ammonium, starch, and soluble sugar concentrations. Among N compounds, protein, total amino acid and ammonium concentrations generally declined in response to reduced N application. On the other hand, accumulation of nitrate increased under lower N supply in all genotypes. The grain starch concentration was slightly elevated in all genotypes except for MD. The soluble sugar concentrations in grains were genotype-dependent. MD, which exhibited the lowest grain yield at low N, contained the highest protein, total amino acids, ammonium, and nitrate, but had the lowest starch and soluble sugars.

Correlation of N and C compounds in grains was analyzed at each N treatment (Tab. 4). Protein was positively correlated with amino acid and ammonium concentrations at all N regimes. However, a positive relationship between amino acid and ammonium concentrations was detected only at high and medium N. It was also observed that total soluble sugar concentration was positively correlated with protein and ammonium concentrations at high N. By contrast, total soluble sugar was adversely related to nitrate at medium N and protein and amino acids at low N.

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Table 1: Effect of N application on yield components, grain protein, and N use efficiency in four soft red winter wheat genotypes.[#]

	Grain weight plant^{-1} (g)			**NUEY**			**Spike number plant^{-1}**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{a}10.2_{a}$	$^{a}9.0_{b}$	$^{ab}7.7_{c}$	$^{a}24.2_{c}$	$^{a}40.1_{b}$	$^{ab}49.3_{a}$	$^{a}5.4_{a}$	$^{a}4.8_{a}$	$^{a}4.6_{a}$
MD05W10208-11-8	$^{a}10.1_{a}$	$^{b}7.6_{b}$	$^{c}6.5_{c}$	$^{a}23.9_{c}$	$^{b}33.9_{b}$	$^{c}41.8_{a}$	$^{a}5.4_{a}$	$^{b}4.1_{b}$	$^{ab}3.9_{b}$
SISSON	$^{a}10.3_{a}$	$^{a}9.0_{b}$	$^{a}8.1_{c}$	$^{a}24.4_{c}$	$^{a}39.9_{b}$	$^{a}52.4_{a}$	$^{b}4.4_{a}$	$^{b}3.8_{b}$	$^{b}3.6_{b}$
OH06-150-57	$^{a}10.3_{a}$	$^{a}9.1_{b}$	$^{bc}7.0_{c}$	$^{a}24.3_{b}$	$^{a}40.6_{a}$	$^{bc}45.0_{a}$	$^{ab}4.6_{a}$	$^{b}4.0_{b}$	$^{b}3.7_{b}$
ANOVA	G**	N**	G × N**	G**	N**	G × N**	G**	N**	G × N ns
	Grain number spike^{-1}			**1000-seed weight (g)**			**Spike length (cm)**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{b}56.9_{a}$	$^{b}53.6_{ab}$	$^{b}47.6_{b}$	$^{bc}36.3_{a}$	$^{bc}36.2_{a}$	$^{a}34.4_{a}$	$^{a}11.5_{a}$	$^{a}11.0_{a}$	$^{a}10.9_{a}$
MD05W10208-11-8	$^{ab}59.0_{a}$	$^{b}55.4_{ab}$	$^{b}50.5_{b}$	$^{c}32.6_{a}$	$^{c}33.9_{a}$	$^{b}33.0_{a}$	$^{d}9.0_{a}$	$^{b}8.9_{a}$	$^{c}8.9_{a}$
SISSON	$^{a}65.3_{a}$	$^{a}62.4_{a}$	$^{a}60.3_{a}$	$^{ab}36.7_{a}$	$^{ab}38.6_{a}$	$^{a}38.4_{a}$	$^{c}9.8_{a}$	$^{b}9.2_{b}$	$^{c}8.8_{b}$
OH06-150-57	$^{ab}62.0_{a}$	$^{ab}58.7_{a}$	$^{b}52.1_{b}$	$^{a}38.9_{a}$	$^{a}41.8_{a}$	$^{a}38.8_{a}$	$^{b}10.5_{a}$	$^{a}10.4_{ab}$	$^{b}9.9_{b}$
ANOVA	G**	N**	G × N ns	G**	N*	G × N ns	G**	N**	G × N ns
	Harvest Index			**Vegetative biomass (g)**			**Grain protein content (%)**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{ab}0.49_{a}$	$^{b}0.47_{b}$	$^{b}0.44_{b}$	$^{a}10.6_{a}$	$^{a}10.1_{a}$	$^{a}9.8_{a}$	$^{ab}14.5_{a}$	$^{bc}10.6_{b}$	$^{b}9.2_{c}$
MD05W10208-11-8	$^{ab}0.49_{a}$	$^{b}0.47_{b}$	$^{b}0.45_{c}$	$^{ab}10.2_{a}$	$^{b}8.5_{b}$	$^{b}8.0_{b}$	$^{a}15.3_{a}$	$^{a}12.2_{b}$	$^{a}10.3_{c}$
SISSON	$^{a}0.52_{a}$	$^{a}0.53_{a}$	$^{a}0.49_{b}$	$^{b}9.4_{a}$	$^{b}8.4_{b}$	$^{b}8.1_{b}$	$^{a}15.5_{a}$	$^{b}11.1_{b}$	$^{b}9.5_{c}$
OH06-150-57	$^{b}0.48_{a}$	$^{b}0.46_{b}$	$^{b}0.44_{c}$	$^{a}11.0_{a}$	$^{a}10.6_{a}$	$^{ab}8.9_{b}$	$^{b}13.7_{a}$	$^{c}10.3_{b}$	$^{b}9.4_{c}$
ANOVA	G**	N**	G × N ns	G**	N**	G × N**	G**	N*	G × N**
	NUEP								
	High	Medium	Low						
IL07-4415	$^{ab}3.6_{b}$	$^{a}4.3_{a}$	$^{a}4.5_{a}$						
MD05W10208-11-8	$^{ab}3.6_{b}$	$^{a}4.1_{a}$	$^{a}4.4_{a}$						
SISSON	$^{a}3.8_{c}$	$^{a}4.5_{b}$	$^{a}4.9_{a}$						
OH06-150-57	$^{b}3.3_{b}$	$^{b}4.3_{a}$	$^{a}4.5_{a}$						
ANOVA	G**	N**	G × N ns						

[#]Superscript and subscript letters represent mean comparisons among four accessions and among three N regimes, respectively. Values not sharing the same letter are significantly different (P < 5%). Results from ANOVA are expressed as: G = Genotype; N = N treatment; G ×N = Genotype × N treatment interaction; ns = not significant; *P < 5%; **P < 1%.

3.4 Accumulation of N compounds in flag leaves under three N regimes

To evaluate the effect of N supply on the accumulation of N compounds in flag leaves at anthesis, the concentrations of protein, total amino acids, ammonium, and nitrate were quantified in the four genotypes (Tab. 5). Two-way ANOVA showed that all components surveyed were significantly influenced by genotypes and N treatment. However, the effect of the genotype × N supply interaction was not significant on amino acid and chlorophyll concentrations. A decline in N application rate reduced the levels of protein, total amino acids, and ammonium in the four genotypes, but the concentration of nitrate was elevated in IL and Sisson in response to lower N. In Sisson, the level of nitrate was highest under low N supply, whereas the abundance of protein, total amino acids, and ammonium was relatively low as compared to the other three

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Table 2: Correlation coefficient analysis of yield components, grain protein concentration, and N use efficiency under each nitrogen condition.[a]

		Grain weight plant^{-1}	NUEY	Spike number plant^{-1}	Grain number spike^{-1}	1000-seed weight	Spike length	Harvest index	Vegetative biomass	Grain protein concentration	NUEP
Grain weight plant^{-1}	H	–									
	M	–									
	L	–									
NUEY	H	1.00***	–								
	M	1.00***	–								
	L	1.00***	–								
Spike number plant^{-1}	H	–0.02	–0.02	–							
	M	0.18	0.18	–							
	L	0.30*	0.30*	–							
Grain number spike^{-1}	H	0.34*	0.34*	–0.74***	–						
	M	0.14	0.14	–0.70***	–						
	L	0.35**	0.35**	–0.66***	–						
1000-seed weight	H	0.36***	0.36***	–0.65***	0.26	–					
	M	0.62***	0.62***	–0.29*	0.05	–					
	L	0.43**	0.43**	–0.34*	0.30*	–					
Spike length	H	0.00	0.00	–0.15	0.02	0.24	–				
	M	0.34**	0.34**	–0.01	0.01	0.39**	–				
	L	0.08	0.08	0.08	–0.13	0.20	–				
Harvest index	H	0.54***	0.54***	–0.36**	0.41**	0.41**	–0.12	–			
	M	0.41**	0.41**	–0.34**	0.39**	0.33*	–0.18	–			
	L	0.48***	0.48***	–0.28*	0.55***	0.32*	–0.39**	–			
Vegetative biomass	H	0.05	0.05	0.40**	–0.24	–0.24	0.18	–0.81***	–		
	M	0.41**	0.41**	0.50***	–0.25	0.18	0.45***	–0.65***	–		
	L	0.69***	0.69***	0.58***	–0.08	0.17	0.39**	–0.30*	–		
Grain protein concentration	H	–0.54***	–0.54***	–0.01	–0.19	–0.35*	–0.27	–0.15	–0.23	–	
	M	–0.56***	–0.56***	–0.13	0.01	–0.46**	–0.54***	0.08	–0.52***	–	
	L	–0.17	–0.17	0.05	0.05	–0.43**	–0.41**	0.25	–0.36*	–	
NUEP	H	0.67***	0.67***	0.11	0.10	0.09	–0.11	0.52***	–0.16	0.27	–
	M	0.30	0.30	–0.11	0.13	0.24	0.07	0.41**	–0.18	–0.02	–
	L	0.42**	0.42**	–0.10	0.35*	0.35*	–0.07	0.45**	0.10	0.21	–

[a]H, M, and L represent high, medium, and low N conditions, respectively. $^{*}P < 5\%$; $^{**}P < 1\%$; $^{***}P < 0.1\%$.

genotypes. The abundance of chlorophyll *a* and *b* was reduced in response to lower N application at distinct rates in these accessions. Under low N input, the concentration of chlorophyll *a* and *b* was lowest in Sisson.

Correlation analysis of these traits was performed at each N regime (Tab. 6). The abundance of total amino acids and ammonium was positively correlated at all N regimes. Nitrate level was negatively related to the accumulation of total amino acids and ammonium under all N conditions. No significant correlation was detected between protein and other N-com-

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Table 3: Effect of N application on the accumulation of nitrogen compounds and carbohydrates in grains of four soft red winter wheat accessions.[#]

	Protein (mg g^{-1})			**Total amino acids (μmol g^{-1})**			**Ammonium (μmol g^{-1})**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{a}146.6_{a}$	$^{b}102.2_{b}$	$^{c}93.2_{c}$	$^{a}30.1_{a}$	$^{c}19.9_{b}$	$^{c}17.9_{c}$	$^{b}839.9_{a}$	$^{c}423.5_{b}$	$^{b}406.7_{b}$
MD05W10208-11-8	$^{a}145.7_{a}$	$^{a}119.8_{b}$	$^{a}116.4_{b}$	$^{a}31.4_{a}$	$^{b}25.1_{c}$	$^{a}26.2_{b}$	$^{b}834.7_{a}$	$^{b}599.2_{c}$	$^{a}655.4_{b}$
SISSON	$^{a}154.4_{a}$	$^{a}116.2_{b}$	$^{b}101.1_{c}$	$^{a}30.5_{a}$	$^{a}27.5_{a}$	$^{b}20.9_{b}$	$^{a}1011.9_{a}$	$^{a}909.8_{b}$	$^{a}637.9_{c}$
OH06-150-57	$^{b}135.2_{a}$	$^{ab}109.2_{b}$	$^{b}101.7_{c}$	$^{b}18.1_{a}$	$^{d}17.4_{a}$	$^{c}16.9_{a}$	$^{c}428.9_{b}$	$^{b}516.2_{b}$	$^{a}670.5_{a}$
ANOVA	G**	N**	G × N ns	G**	N**	G × N**	G**	N**	G × N**
	Nitrate (μmol g^{-1})			**Starch (mmol g^{-1})**			**Soluble sugars (μmol g^{-1})**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{a}9.8_{c}$	$^{a}11.3_{b}$	$^{a}13.2_{a}$	2.8_{b}	2.8_{b}	3.2_{a}	$^{b}227.8$	$^{b}218.9$	$^{a}245.3$
MD05W10208-11-8	$^{b}7.1_{b}$	$^{ab}10.7_{a}$	$^{ab}12.6_{a}$	2.9_{a}	2.9_{a}	2.7_{a}	$^{bc}209.6$	$^{b}205.9$	$^{c}204.8$
SISSON	$^{b}7.1_{b}$	$^{bc}9.1_{a}$	$^{c}8.6_{a}$	2.7_{b}	2.9_{ab}	3.1_{a}	$^{a}260.6$	$^{a}248.8$	$^{b}226.6$
OH06-150-57	$^{ab}7.4_{b}$	$^{c}8.3_{b}$	$^{b}11.7_{a}$	2.7_{b}	2.9_{b}	3.5_{a}	$^{c}189.2$	$^{a}244.7$	$^{a}249.6$
ANOVA	G**	N**	G × N ns	G ns	N**	G × N*	G**	N ns	G × N**

[#]Superscript and subscript letters represent mean comparisons among four accessions and among three nitrogen regimes, respectively. Values not sharing the same letter are significantly different (P < 5%). Results from ANOVA are expressed as: G = Genotype; N = N treatment; G × N = Genotype × N treatment interaction; ns = not significant; *P < 5%; **P < 1%.

pounds at any N regime. However, protein concentration was positively and strongly correlated with the accumulation of chlorophyll *a* and *b* but only at low N.

4 Discussion

This study evaluated N responses of grain yield, grain protein content, NUE, and their associated traits in four soft red winter wheat genotypes. The responsiveness of yield components to environmental factors has been evaluated in cereals including wheat (*Sadras* and *Slafer*, 2012; *Slafer* et al., 2014). According to their analyses, stress responsiveness of spike number plant^{-1} is generally similar to that of grain number spike^{-1}. However, their responsiveness is more sensitive than that of grain weight. Consistently, this study demonstrates that spike number plant^{-1} and grain number spike^{-1} significantly declined in response to reduced N supply in most accessions, whereas a reduction in N supply did not change 1000-seed weight in any of the genotypes (Tab. 1). It has been confirmed that responsiveness of yield components to environmental factors is inversely associated with their heritability in wheat and other cereals (*Sadras* and *Slafer*, 2012). Due to its high heritability, grain weight may be a major contributor for genotypic variation in grain yield at variable N rates. The 1000-seed weight was positively correlated with grain weight plant^{-1} (equivalent to grain yield) at all N rates (Tab. 2). By contrast, correlation of spike number plant^{-1} and grain number spike^{-1} with grain weight plant^{-1} was not significant at higher N levels, presumably due to lower heritability of these traits. These results suggest that 1000-seed weight (grain weight) can be a key component determining grain yield irrespective of N availability.

Grain protein concentration is a critical trait associated with the milling and baking quality of wheat grains. Consistent with other field data (*Kibite* and *Evans*, 1984; *Oury* and *Godin*, 2007; *Bogard* et al., 2010), this study indicated that grain protein concentration was inversely associated with grain weight plant^{-1} and NUEY at high and medium N (Tab. 2). These negative relationships have made it difficult to improve grain yield and grain protein concentration simultaneously (*Bogard* et al., 2010). Grain protein and nitrogen concentrations decreased with the year of cultivar release in Italian and Spanish winter wheat (*Guarda* et al., 2004; *Acreche* and *Slafer*, 2009). For sustainable wheat production, NUEP is an important trait to reduce N usage per unit area while maintaining grain protein concentration. Interestingly, this study revealed a positive relationship between NUEP and NUEY at high and low N (Tab. 2). Both of these NUE traits were positively associated with grain number spike^{-1}, 1000-seed weight, and harvest index at low N. These yield-associated components may serve as proxy traits to select high NUEY and NUEP genotypes in soft red winter wheat.

Flag leaves provide the majority of assimilates for grains during post-anthesis and grain filling stages (*Simpson* et al., 1983). It was shown that increased N supply results in increased leaf area, leaf N and chlorophyll concentrations in flag leaves of wheat (*Evans*, 1983). Conversely, N limitation reduces the size of leaves due to lower cell numbers and

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Table 4: Correlation coefficient analysis of N and C compounds in grains under each N regime.[a]

		Protein	Amino acids	Ammonium	Nitrate	Starch	Total soluble sugars
Protein	H	–					
	M	–					
	L	–					
Amino acids	H	0.70*	–				
	M	0.72**	–				
	L	0.75**	–				
Ammonium	H	0.80**	0.88***	–			
	M	0.65*	0.82***	–			
	L	0.62*	0.36	–			
Nitrate	H	–0.18	0.18	0.14	–		
	M	–0.26	–0.06	–0.35	–		
	L	0.05	–0.14	–0.47	–		
Starch	H	–0.31	–0.54	–0.32	–0.13	–	
	M	0.22	–0.14	0.34	–0.41	–	
	L	–0.05	–0.34	0.43	–0.03	–	
Total soluble sugars	H	0.79**	0.50	0.69*	–0.19	0.13	–
	M	0.05	–0.03	0.39	–0.61*	0.56	–
	L	–0.73**	–0.64*	–0.26	0.01	0.35	–

[a]H, M, and L represent high, medium, and low nitrogen input, respectively. *P < 5%; **P < 1%; ***P < 0.1%.

volume together with protein and chlorophyll concentrations (*Lawlor* et al., 1989). Consistent results were observed in this study where increased N supply raised protein and chlorophyll concentrations in flag leaves at anthesis (Tab. 5). In addition, increasing N supply also resulted in greater accumulation of total amino acids and ammonium, which were positively correlated under all N regimes (Tab. 6). The effect of N supply on nitrate accumulation in flag leaves varied among genotypes. Nevertheless, the level of nitrate was inversely and strongly associated with amino acid and ammonium accumulation at all N regimes. It seems that the reduction rates of nitrate and nitrite into ammonium are critical determinants for the concentrations of ammonium and total amino acids in flag leaves, regardless of N rates. We also observed that protein concentration was adversely correlated with the levels of chlorophyll *a* and *b* but only at low N. This result suggests that protein and chlorophyll biosynthesis compete with each other when N availability is limited in flag leaves.

Consistent with the observations in flag leaves, limited N supply considerably restricted the accumulation of major N compounds such as protein, total amino acids and ammonium in grains (Tab. 3). These N compounds were positively correlated with each other under all N regimes except for the correlation between ammonium and total amino acids at low N (Tab. 4). In contrast to N compounds, the levels of C compounds, such as starch and soluble sugars, were stable or only slightly altered at variable N rates (Tab. 3). It seems that N supply does not largely affect grain carbohydrate concentrations in soft red winter wheat. Limitation of N availability reduces chlorophyll, protein and photosynthesis-related enzyme (*e.g.*, Rubisco) concentrations in flag leaves (*Evans*, 1983; *Lawlor* et al., 1989; *Zhu* et al., 2010), which can result in reduced performance in the biosynthesis of both C and N compounds.

A large decline in protein concentration and stabilized carbohydrate level in grains at low N can be attributed to distinct energy costs required for protein, amino acid, soluble carbohydrates, and starch synthesis. Production of protein and amino acids requires more energy and carbon costs than that of soluble carbohydrates and starch (*Vertregt* and *Penning de Vries*, 1987; *Munier-Jolain* and *Salon*, 2005). Reduced accumulation of protein and other N compounds in grain may be an energy-saving strategy to maintain carbohydrate synthesis and minimize yield loss under N-limited conditions. It has also been demonstrated that grain protein concentration is positively and strongly correlated with total soluble sugar concentration at high N, whereas these two components are adversely associated at low N (Tab. 4). This result indicates that trade-off between protein and soluble carbohydrate

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Table 5: Effect of nitrogen application on the accumulation of N compounds and chlorophylls in flag leaves of four soft red winter wheat accessions.[#]

	Protein (mg g^{-1} FW)			**Total amino acids (μmol g^{-1} FW)**			**Ammonium (μmol g^{-1} FW)**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
	$^{ab}20.5_a$	$^{a}13.5_b$	$^{b}9.0_c$	$^{a}43.7_a$	$^{a}33.8_b$	$^{b}30.0_c$	$^{a}5.4_a$	$^{a}3.3_b$	$^{a}2.5_c$
MD05W10208-11-8	$^{b}18.4_a$	$^{b}8.6_c$	$^{a}12.9_b$	$^{a}44.6_a$	$^{a}33.7_b$	$^{a}32.0_b$	$^{a}5.5_a$	$^{a}3.2_b$	$^{a}2.5_c$
SISSON	$^{ab}19.6_a$	$^{a}13.3_b$	$^{b}9.0_c$	$^{b}35.7_a$	$^{b}27.2_b$	$^{c}23.1_c$	$^{c}1.5_a$	$^{b}1.6_a$	$^{c}1.0_b$
OH06-150-57	$^{a}21.1_a$	$^{a}14.3_b$	$^{a}14.5_b$	$^{b}33.5_a$	$^{b}26.9_b$	$^{c}23.0_c$	$^{b}2.4_a$	$^{b}1.8_b$	$^{b}1.5_c$
ANOVA	G**	N**	G × N**	G**	N**	G × N ns	G**	N**	G × N**
	Nitrate (μmol g^{-1} FW)			**Chlorophyll a (μmol g^{-1} FW)**			**Chlorophyll b (μmol g^{-1} FW)**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{d}12.1_b$	$^{c}15.7_a$	$^{c}16.9_a$	$^{a}2.2_a$	$^{sb}2.0_b$	$^{bc}1.8_b$	$^{b}0.46_a$	$^{b}0.42_a$	$^{c}0.36_a$
MD05W10208-11-8	$^{c}18.0_{ab}$	$^{b}20.4_a$	$^{c}16.5_b$	$^{a}2.3_a$	$^{a}2.2_{ab}$	$^{a}2.1_b$	$^{b}0.52_a$	$^{ab}0.52_a$	$^{b}0.44_b$
SISSON	$^{b}31.7_c$	$^{a}42.2_b$	$^{a}61.2_a$	$^{a}2.2_a$	$^{ab}2.1_a$	$^{c}1.7_b$	$^{b}0.46_a$	$^{ab}0.44_a$	$^{c}0.33_b$
OH06-150-57	$^{a}37.4_a$	$^{b}23.8_b$	$^{b}21.1_c$	$^{a}2.3_a$	$^{b}1.9_b$	$^{b}2.0_b$	$^{a}0.64_a$	$^{a}0.53_b$	$^{a}0.53_b$
ANOVA	G**	N*	G × N**	G**	N**	G × N ns	G**	N**	G × N ns

[#]Superscript and subscript letters represent mean comparisons among four accessions and among three nitrogen regimes, respectively. Values not sharing the same letter are significantly different (P < 5%). Results from ANOVA are expressed as: G = Genotype; N = N treatment; G × N = Genotype × N treatment interaction; ns = not significant; *P < 5%; **P < 1%.

synthesis is not necessary when a sufficient amount of N is available, but production of these compounds competes with each other under N limitation.

5 Conclusions

Our results indicate that high heritability traits such as 1000-seed weight and harvest index were consistently associated with grain weight plant^{-1} (equivalent to grain yield) and NUEY regardless of N regimes. In addition, positive relationships between NUEY and NUEP were observed at low N, both of which are correlated with grain number spike^{-1}, 1000-seed weight, and harvest index. Selection of these yield components under N-limited conditions can lead to the simultaneous enhancement of NUEY and NUEP in soft red winter wheat.

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Table 6: Correlation coefficient analysis of N compounds and chlorophyll concentrations in flag leaves under each N regime.[a]

		Protein	Amino acids	Ammonium	Nitrate	Chlorophyll a	Chlorophyll b
Protein	H	–					
	M	–					
	L	–					
Amino acids	H	–0.04	–				
	M	–0.50	–				
	L	0.01	–				
Ammonium	H	–0.17	0.83***	–			
	M	–0.49	0.91***	–			
	L	0.16	0.87***	–			
Nitrate	H	0.28	–0.80***	–0.82***	–		
	M	0.19	–0.69*	–0.87***	–		
	L	–0.46	–0.62*	–0.89***	–		
Chlorophyll a	H	0.10	0.13	0.26	–0.06	–	
	M	–0.53	0.25	0.13	0.13	–	
	L	0.71***	0.47	0.56	–0.70*	–	
Chlorophyll b	H	0.17	–0.38	–0.20	0.56	0.42	–
	M	–0.25	0.02	0.13	–0.11	0.76***	–
	L	0.82***	–0.07	0.24	–0.58*	0.79***	–

[a]H, M, and L represent high, medium and low nitrogen input, respectively. *P < 5%; **P < 1%; ***P < 0.1%.

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