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Effects of cultivars and nitrogen management on wheat grain yield and protein

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Core Ideas

1. Grain yield was associated positively with the water supply to demand ratio in the season.
2. There was no yield advantage to spring or split N application over fall except for 1 site-year.
3. Application of N increased winter wheat grain protein in both dry and wet years.
4. In site-years with low water supply and demand ratio, protein was inversely related to yield.
5. Fertilizer N Recovery Efficiency decreased with an increase in applied N rates.

ABSTRACT

Low grain protein in hard red winter (HRW) wheat (*Triticum aestivum* L.) is a serious challenge for rainfed wheat growers, particularly in years with elevated grain yield. Proper nitrogen (N) management with adequate N rate and application timing is critical for optimizing grain yield and protein content. This 2-yr experiment evaluated the effects of different N rates and application timings (fall, spring, and split) on grain yield and protein of two HRW wheat cultivars. Field studies were conducted at four different sites across Nebraska under rainfed conditions in 2018/2019 (Year 1) and 2019/2020 (Year 2). A split-plot randomized complete block design with wheat cultivars as the whole plots and factorial combinations of six N rates and three application timings as the sub-plots was used in four replications. Grain yield was associated positively and grain protein negatively with the water supply to demand ratio (WS:WD) in the season. Freeman cultivar yielded better in a year with higher WS:WD and a newly developed Ruth yielded better in a lower WS:WD year. Nitrogen fertilization significantly increased grain yield in the site-years with moderately higher WS:WD. There was an increase in grain protein with increasing N rates at all site-years. Spring and split applied N resulted in better grain yield than fall application in the site-year when there was a risk of N loss. This experiment suggested that an effective N management strategy for winter wheat should account for and be adaptable to weather variability to optimize grain yield and protein content.

Keywords: Nitrogen management, dryland winter wheat, wheat protein

Abbreviations: AOR, agronomic optimum rate; GS, growth stage; HRW, hard red winter; N, nitrogen

INTRODUCTION

Winter wheat (*Triticum aestivum* L.) is an important crop in terms of production and market value in the U.S. Great Plains (Weiss et al., 2003). Among six classes of wheat grown in the U.S., hard red winter (HRW) wheat accounts for 40% of total wheat production in the U.S. (Tilley et al., 2012). Hard red winter wheat is commonly used in the preparation of a wide range of flour-based products due to its moderately high protein content (120 – 140 g kg^{-1}) (Gibson & Newsham, 2018). Grain protein content is an important quality factor of HRW wheat for its end-use functionality in the milling and baking industry (Fuertes-Mendizabal et al., 2013; Shewry, 2007; Fufa et al., 2005; Maghirang et al., 2006). The drop in grain protein below the threshold results in reduced revenue for HRW wheat producers as industries set up price adjustments based on the protein content (Woolfolk et al., 2002). Conversely, increasing grain protein content in HRW wheat can benefit wheat producers with additional premiums in grain prices (Dick et al., 2016).

Low grain protein in HRW wheat has been a serious concern among wheat growers and wheat industry, particularly in years with elevated grain yield. Regional quality survey reported a significantly lower average protein content in HRW wheat for 2016 (112 g kg^{-1}) and 2017 (111 g kg^{-1}) compared to preceding two five-year averages (125 g kg^{-1} and 123 g kg^{-1}) (Plains Grains Inc., 2018).

Grain yield and protein content in wheat are affected by several factors including climate, cultivar, nitrogen (N) management, and soil properties (Peterson et al., 1988, Wilson & Gallagher, 1990). Nitrogen is one of the most important management factors affecting grain yield and protein in wheat (Zorb et al., 2018; Wilson et al., 2020; Cassman et al., 1992). Determining optimal N rate and application timing is critical for both yield and grain protein. Grain protein content in wheat can be increased by applying N at rates greater than agronomic optimum rate (AOR). As yield reaches its maximum with AOR, the additional N contributes to increasing grain protein (Dick et al., 2016; Walsh et al., 2018; Macy, 1936; Goos et al., 1982; Lollato et al., 2019). However, an increase in grain protein due to additional N may vary by N application timing (Nakano et al., 2008; Romero et al., 2017). Reports suggest that N applied at or before sowing may result in little or no effect on grain protein content (Graham and Stockton, 2019). In contrast, N applied at heading or post heading stage is found to substantially increase grain protein content (Cooper, 1974; Cruppe et al., 2017; Lollato et al., 2021).

Achieving an adequate protein content and increase in grain yield simultaneously is challenging for wheat producers. Several previous studies reported a negative correlation between grain protein content and grain yield in wheat (Simmonds, 1995; Triboi et al., 2006; Oury & Godin, 2007). The rainfed production system makes it more challenging since it receives annual precipitation lower than annual potential water evapotranspiration (Stewart, 2016). Some studies reported improved grain yield and grain protein content with a considerably high fertilizer N rate in a rainfed environment (Brown et al., 2005; Romero et al., 2017). In Oklahoma under rainfed conditions with an average annual precipitation of 874 mm, Mohammed et al. (2013) reported a linear increase in grain yield along with higher grain protein with an increase in N rates up to 200 kg N ha^{-1}. Application of N rate greater than AOR targeting higher grain yield and grain protein can increase the chance of lodging and thus, negatively affect wheat grain yield and quality (Shah et al., 2019; Zhang et al., 2017). In addition, wheat response to N rate and application timing varies across years and environmental conditions (Mohammed et al., 2013). Therefore, determining the optimum rate and timing of N application that are specific to a region and that account for weather is important from agronomic, economic, and environmental perspectives.

Grain yield, grain protein content, and their relationships may differ by cultivars besides various other factors including N rates, N application timing, N availability, and growing environment (Gaju et al., 2014; Ortiz-Monasterio et al., 1997; Latshaw et al., 2016; Triboi et al., 2006). Differences in anthesis date, grain filling duration, kinetics of canopy senescence, N uptake and assimilation, post-anthesis N remobilization efficiency, etc. among cultivars can result variation in grain yield and grain protein content as well as N use efficiency (Hawkesford, 2017; Foulkes et al., 2009; Barraclough et al., 2014). Currently, ‘Ruth’ and ‘Freeman’, HRW wheat cultivars adapted to dryland environment, are two widely sowed cultivars in Nebraska (Nebraska Wheat Board, 2021). To our knowledge, they are yet to be evaluated together and compared in terms of grain yield, grain protein content, and N use efficiency in different environments and under varying N management.

The objective of this experiment was to evaluate the effects of different N rates and application timing on grain yield and protein content of two cultivars of HRW wheat (Ruth and Freeman) across Nebraska. We hypothesized that higher rates and split application of N would be optimal to maximize winter wheat grain yield along with adequate protein content given an adequate soil moisture.

MATERIALS AND METHODS

Experimental Sites

In 2018/2019 (Year 1) and 2019/2020 (Year 2), field experiments were conducted under rainfed conditions at four different research stations located across Nebraska: Eastern Nebraska Research and Extension Center near Mead (ENREC, 41.16˚N, 96.41˚W, elevation 348 m), Henry J. Stumpf International Wheat Center near Grant (GRANT, 40.85˚N, 101.71, elevation 1037 m), High Plains Agricultural Lab near Sidney (HPAL, 41.23˚N, 103.00˚W, elevation 1310 m), and Panhandle Research and Extension Center near Scottsbluff (PREC, 41.89˚N, 103.68˚W, elevation 1197 m). The 30-yr average precipitation (1980 – 2010) during winter wheat growing season at ENREC, GRANT, HPAL, and PREC are 653 mm, 463 mm, 377 mm, and 360 mm respectively. The soils at ENREC were Filbert silt loam (fine, smectitic, mesic Vertic Argialboll) in Year 1 and Tomek silt loam (fine, smectitic, mesic Pachic Arguidoll) in Year 2. At GRANT, soil was Mace silt loam (fine-silty, mixed, superactive, mesic Aridic Arguistoll) in Year 1 and Kuma silt loam (fine-silty, mixed, superactive, mesic Pachic Argiustoll) in Year 2. The predominant soil at HPAL was Duroc loam (fine-silty, mixed, superactive, mesic Pachic Haplustoll) in Year 1 and Alliance loam (fine-silty, mixed, superactive, mesic Aridic Argiustoll) in Year 2. At PREC, soil was Tripp very fine sandy loam (coarse-silty, mixed, superactive, mesic Aridic Haplustoll) in both years (USDA-NRCS, 2020). The experimental fields for two years at each research station were located within less than a kilometer apart. Daily precipitation and reference evapotranspiration (ET_o) data for each winter wheat growing season were obtained from weather stations located near the experimental sites (HPRCC, 2020). In-season cumulative precipitation was considered as seasonal water supply (WS) and ET_o as the seasonal water demand (WD).

For each site-year, the experimental layout was split-plot randomized complete block design with four replications. The main plot factor was wheat cultivar. Two HRW wheat cultivars, 'Ruth' (Reg. no. CV-1165, PI 675998; Baenziger et al., 2020) and 'Freeman' (Reg. No. CV- 1098, PI 667038; Baenziger et al., 2014) were selected based on their wide use among farmers, broad adaptability, and representation of distinctly different breeding lineages. A factorial combination of three fertilizer N application timings and six N rates were randomly assigned to the split plots. Three N application timings were 100% in fall, 100% in spring, and split (30% in fall and 70% in spring) and six N rates were 0%, 25%, 50%, 75%, 100%, and 125% of recommended N rate at each site. The 0% N rate is hereafter referred to as the control treatment. The recommended N rate was calculated using Winter Wheat Fertilizer Calculator from the University of Nebraska-Lincoln (UNL) (Hergert and Saver, 2009) that accounts for nitrate-N from pre-plant soil test (0-120 cm) and fertilizer N and grain prices. The UNL algorithm outputs N rate for yield goal up to 5.04 Mg ha^{-1} and recommends an additional 23 kg N ha^{-1} for yield goal above 5.04 Mg ha^{-1}. The three-year average yield (2015-2017) from Nebraska Statewide Winter Wheat Variety Trials for all study locations was less than 5.04 Mg ha^{-1} (NE Winter Wheat Variety Test, 2019).

Considering for yield goal > 5.04 Mg ha^{-1} and fertilizer N price at \$0.88 per kg, and wheat grain price at \$0.165 per kg, recommended N rate was 67 kg N ha^{-1} at GRANT, HPAL, and PREC. At ENREC, the recommended N rate was 90 kg N ha^{-1}, greater than at other three sites considering a greater yield potential (>5.04 Mg ha^{-1}) due to higher seeding rates and better growing conditions. The actual rates of fertilizer N used for different N rate treatments are detailed in Supplementary Table 1.

Ammonium nitrate (34-0-0) was used as the N source and was manually surface broadcast in the plots. The fall portion of N (both in split and fall treatments) was applied approximately two weeks after sowing. Feekes growth scale (GS), a common scale for staging wheat growth, was used as a guide for subsequent N application (Large, 1954). The spring N application in split and spring treatments was applied near Feekes GS 5 (late tillering) stage of wheat. Fungicide (active ingredients: prothioconazole and tebuconazole at 0.007 and 0.009 g ha^{-1}, respectively) was applied at early flowering stage (Feekes GS 10.5.1) at GRANT in Year 1 and at ENREC in Year 2.

In Year 1, the trials were planted on Oct 4, Sept 12, Sept 11, and Sept 17 of 2018 at ENREC, GRANT, HPAL, and PREC respectively using plot drills (Almaco, IA, U.S. at ENREC, an SRES drill, Seed Research Equipment Solutions, KS, U.S. at GRANT and HPAL, and a custom-built drill with a Hege cone at PREC). The sowing dates for Year 2 trials were Sept 26, Sept 17, Sept 16, and Sept 24 of 2019 at ENREC, GRANT, HPAL, and PREC, respectively. In both years, a seeding rate of 2.44 million seeds ha^{-1} was used at ENREC while a seeding rate of 2.06 million seeds ha^{-1} was used at GRANT, HPAL, and PREC. The dates for fertilizer application and harvest are presented in Figure 1.

Field Data Collection

At each location, pre-sowing soil samples were collected one to seven days before sowing at three to five random spots across the experimental field to a depth of 120 cm at three intervals (0-20, 20-60, and 60-120 cm). Each soil sample was analyzed for nitrate-N (NO_3-N) and other chemical properties (Table 1). Post-harvest soil samples were collected within a week of harvest to a depth of 90 cm from selected plots with N rates (0%, 75%, 100%, and 125%) from all application timing for Ruth cultivar and analyzed for NO_3-N. All soil tests were done using standard procedures (Ward Laboratories Inc., Nebraska, U.S.). At PREC in Year 1, 50 kg P ha^{-1} was uniformly applied across the field as soil P test recommended for it (Hergert and Shaver, 2009).

Winter wheat grains were harvested at maturity from each plot with SPC40 (Almaco, IA, U.S.) at ENREC, Zurn$^{©}$ 150 universal plot combine harvester (Zurn harvesting GmbH & Co. KG, U.S.) at GRANT and HPAL, and with a Delta plot combine (Wintersteiger Inc., UT, U.S.) at PREC. The average yield per plot and moisture percentage of grains were recorded by the weigh system on the plot combine and the reported yield was adjusted to 12 % moisture. A sub-sample of harvested grain from each plot was collected and analyzed for grain protein content using DA 7250^{TM} NIR analyzer (Perten Instruments, Inc, IL, USA). The grain protein content measurement using NIR follows AACC International Method 39-25.01. The NIR analyzer uses novel Diode Array technology and relates reference values and the spectra of samples using multivariate calibrations. Grain protein content was reported on 12 % moisture basis, the standard used for grain marketing (Wheat Marketing Center, Inc., 2004).

Nitrogen Indices

Grain N concentration was calculated from grain protein content using a conversion factor of 5.83 (Merril & Watt, 1973). Grain N removal (GNR), Nitrogen Use Efficiency as Partial Factor Productivity (PFP), and Nitrogen Recovery Efficiency (NRE) were calculated using the following equations:

$GNR\ (kgN\ ha^{-1}) = \ Grain\ Yield\ x\ Grain\ N\ concentration$ (1) (Lollato et al., 2021

$PFP\ (kg\ kg^{-1}) = \frac{Grain\ Yield}{N_{available}}$ (2) (Cassman et al., 1998)

$NRE\ (kg\ kg^{-1}) = \frac{GNR_{N1} - GNR_{N0}}{N_{fertilizer}}$ (3) (Varvel and Peterson, 1990)

where N1 and N0 in the sub-script represent the values of variables from each fertilized subplot and unfertilized subplot, respectively; $N_{available}$ is the total N available in the growing season (pre-sowing soil NO_3-N plus applied fertilizer N); $N_{fertilizer}$ is the amount of N applied as inorganic fertilizer in individual subplots.

Statistical Analysis

Effects of site-year, cultivar, N rate, and N application timing on grain yield and protein were determined using Proc Mixed in SAS 9.4 Software (SAS Institute, 2013) with site nested in year, cultivar, N rate, and N timing as the fixed effects and block and all interactions of block with other terms as random effects. The experimental sites ENREC, GRANT, HPAL, and PREC when referred to as site-years were represented as ENREC18, GRANT18, HPAL18, and PREC18, respectively for Year 1 and as ENREC19, GRANT19, HPAL19, and PREC19, respectively for Year 2. Site-year was treated as fixed effects to examine effects of differences in precipitation patterns between the growing seasons and across sites. In addition, effects of cultivar, N rate and application time, and their interactions on response variables (grain yield, grain protein, GNR, NRE, and PFP) were determined separately for individual site-years. The treatment means were calculated using LSMEANS statement and their differences were compared using DIFF option. Differences were considered significant at $P < 0.05$.

When there was a significant interaction effect of main factors on measured variables, the highest order of interaction effect (3-way>2-way>main factor effect) was only discussed. A pairwise comparison of means by cultivar in the control treatment at different site-years was carried out in SAS using PROC GLM to compare the cultivars. Environmental index (EI) values were calculated by averaging variables across cultivars as described in Lollato et al. (2021) to explain the cultivar by environment interaction effect on grain yield and grain protein content using the concept of adaptability and stability (Eberhart and Russell, 1966).

Linear regression relationships of total available N with grain yield and grain protein content as well as grain yield with grain protein content and GNR were analyzed for each site-year using PROC REG. The residuals from the linear regression of grain protein content against grain yield (referred as GPD; Grain Protein Deviation hereafter) and GNR against grain yield (GNRD: Grain Nitrogen Recovery Deviation) were used to determine the difference between cultivars, N rate, and N application timings in terms of each relationship (Monaghan et al., 2001).

RESULTS

Weather

Total annual precipitation for the wheat growing season in Year 1 (September through July) ranged from 429 mm at PREC18 located in the western region to 815 mm at ENREC18 located in the Eastern region of Nebraska (Figure 1). In Year 2, the total annual precipitation ranged from 260 mm at PREC19 to 554 mm at ENREC19. All sites had greater total precipitation in Year 1 and a lower total precipitation in Year 2 compared to the 30-yr normal. In Year 1, the total annual precipitation was 25, 16, 61, and 19 % greater than the normal precipitation at ENREC, GRANT, HPAL, and PREC, respectively. In Year 2, ENREC, GRANT, HPAL, and PREC had 15, 26, 21, and 28 % lower total annual precipitation than the normal precipitation, respectively. The cumulative growing season ET_o ranged from 975 mm at ENREC18 to 1628 mm at HPAL19 resulting in WS:WD of 0.18 to 0.84 (Figure 1). Year 2 had WS:WD half of that in Year 1. The trial at PREC in Year 1 was completely lost to a series of hailstorms. At ENREC in Year 1, there was a severe infection of fusarium head blight during grain filling stage.

Cultivar differences

Cultivars differed significantly in grain yield at four out of seven site-years (Table 2). Ruth had significantly greater grain yield, GPD, GNR, and PFP at ENREC19 and PREC19 (both in Year 2 with lower WS:WD). Freeman had greater grain yield and PFP at ENREC18 and HPAL18 and greater GNR at HPAL18 (all in Year 1 with greater WS:WD). Grain protein content was greater with Ruth at ENREC18 and GRANT18 and with Freeman at HPAL19.

Adaptability coefficients for two cultivars were similar in terms of grain yield (α = 1.01 for Freeman and 0.989 for Ruth) as well as grain protein (α = 1.03 for Freeman and 0.967 for Ruth, Figure 2). Grain yield and grain protein content were stable for both cultivars (r^2 >0.94).

Agronomic Responses

When responses of grain yield and protein content to N management and cultivar were analyzed with all seven site-years together, significant interaction effects of site-year with cultivar and N rate were observed (Table 3). Irrespective of cultivar, the site-year GRANT18 had the greatest grain yield and HPAL19 had the lowest among site-years. Grain yield or protein did not vary by N application time. Because of the interaction effects involving site-year as well as the main factor effect of site-year on grain yield and protein content, data analyses were conducted for individual site-years separately and are more elaborately discussed.

The regressions of grain yield and grain protein content against WS:WD ratio suggested that grain yield tends to increase linearly (slope = 2.27) as the water supply and demand ratio increases while grain protein content decreased quadratically with an increase in WS:WD and plateaued at WS:WD of 0.43 (Figure 3). All three sites in year 2 had a lower WS:WD ratio and resulted in lower grain yield and higher grain protein content as compared to the sites in year 1 with comparatively higher WS:WS ratio.

Grain Yield at Individual Site-Years

Regression analysis showed a significant linear increase in grain yield with available N at GRANT18, HPAL18, ENREC19, and HPAL19 (Figure 4). The slope of regression was 0.021 Mg per kg available N at HPAL18 compared to 0.011 at GRANT18, 0.004 at ENREC19, and 0.003 at HPAL19. Grain yield at ENREC18 was negatively correlated to available N with a slope of -0.003. In Year 2, grain yield response to available N was not significant at GRANT and PREC.

There was a significant interaction effect of cultivar and N rate on grain yield at ENREC19 and PREC19 (Table 4). At ENREC19, grain yield ranged from 3.32 Mg ha^{-1} with Freeman (the control treatment) to 4.71 Mg ha^{-1} with Ruth (the 125% N treatment) (Figure 5a). At ENREC19, grain yield with Ruth was always greater than Freeman at each of the applied N rates including the control. Within each cultivar, grain yield response to N rates was different. With Ruth, the 125% N rate treatment had significantly greater grain yield than the rest of N rates whereas, with Freeman, the high N rates (50%, 75%, 100%, and 125% N rates) had greater yield than the control treatment and 25% N rate.

At PREC19, grain yield with Ruth was always greater than Freeman at each of the applied N rates except the control (Figure 5b). With Freeman, grain yield with the 25% N rate was significantly greater than with higher N rates except for the 125% N rate. With Ruth, grain yield did not vary by N rate except that the 50% N rate had greater grain yield than the control.

There was a significant cultivar by N application timing interaction effect on grain yield at ENREC19 (Figure 5c). For Ruth, there were greater yields with split and spring N applied treatments compared to the fall treatment. In contrast, no yield difference by N application timing was observed with Freeman.

Cultivar had a significant effect on grain yield at five out of seven site-years (Table 4). Grain yield was significantly greater for Ruth compared to Freeman at GRANT19, ENREC19, and PREC19, all in Year 2, while Freeman yielded significantly greater than Ruth at ENREC18 and HPAL18 both in Year 1 (Table 4).

No significant effect of N application timing on grain yield was observed at site-years except at GRANT18, where the split and spring N applied plots had significantly greater yield compared to fall-applied treatments (Table 4).

Grain yield varied by N rate at GRANT18, HPAL18, and ENREC19 (Table 4), 3 out of 4 site-years where yield had a positive linear correlation with available N rates. Grain yields were in the order 125% >100% =75% >50% =25% >0% at GRANT18 and 125% >100% > 75% =50% > 25% = 0% at HPAL18. Grain yield at N rates of 125, 100, and 75% were greater than the yield at 50, 25, and 0% at ENREC19. Nitrogen rate did not have any significant effect on grain yield at ENREC18, GRANT19, HPAL19, and PREC19.

Grain Protein

Linear regression of grain protein content against available N was significantly positive at all site-years at $P < 0.05$ and at GRANT18 at $P < 0.10$ (Figure 6). The slopes of regression were between 0.0384 and 0.163 g kg^{-1} protein per kg available N. Slopes were greater in Year 2 than in Year 1 for HPAL and GRANT and the reverse was true for ENREC.

There was a significant interaction effect of cultivar by N rate on grain protein content at HPAL18 (Table 5), where Ruth had significantly greater protein content than Freeman at N rates other than the control and 125% N (Figure 7). Grain protein content varied by different N rates with Freeman. The 125% N rate treatment had greater grain protein content than the rest of the N rates. The control and 100% N treatment had greater grain protein content than the 25% treatment. Grain protein content did not vary by N rate with Ruth.

There was a significant N application timing by N rate interaction effect on grain protein content at HPAL19 (Table 5). Among fall-applied treatments, the 125% N rate had significantly greater grain protein content than any other N rates but 100% N (Figure 8). The 100% N rate had greater grain protein content than the control and the 25% N rate. Among split applied treatments, the higher N rates (125%, 100%, and 75% N) had greater grain protein content than the control and the 50% N rate. Among spring-applied treatments, the 125% and 100% N rates had greater grain protein content than in the control and the 25% N rate. Grain protein content at any N rate did not vary by N application time.

The main factor effect of cultivar on grain protein content was significant at HPAL in both Year 1 and 2 (Table 5). In Year 1 when Freeman had greater grain yield than Ruth, the effect of cultivar on grain protein content was reversed (Freeman < Ruth) that year. In Year 2, where yield did not vary by cultivar, grain protein content was significantly greater with Freeman than Ruth. Nitrogen application timing did not have any significant effect on grain protein content at any of the site-years.

A significant effect of N rate on grain protein content was observed at all site-years (Table 5). The plots applied with a higher N rate had greater protein compared to low or no N applied plots. The highest protein content was always below 102 g kg^{-1} at GRANT and HPAL in Year 1. In contrast, greater grain protein content (>102 g kg^{-1}) were achieved at all N applied plots at ENREC in Year 1 with the highest (116 g kg^{-1}) in plots applied with 125 %

N rate. In Year 2, all sites had grain protein content >102 g kg^{-1} irrespective of N rate and as high as 161 g kg^{-1} at GRANT19.

Other Agronomic Responses (GNR, NRE, and PFP)

There was a significant interaction effect of cultivar and N rate on GNR at ENREC19 and PREC19 (Table 6). Ruth tended to have greater GNR (57.1 to 75.3 kg N ha^{-1}) than Freeman (46.8 to 61.3 kg N ha^{-1}) across different N rates. A significant interaction effect of cultivar and N application timing was observed at ENREC19. Ruth had greater GNR compared to Freeman for all N application timings (65.7 to 71 vs 55 to 55.7 kg N ha^{-1}) and GNR differed significantly by N timings for only Ruth with greater GNR for Spring and Split N timings compared to Fall. The main factor effect of cultivar on GNR was significant at ENREC18 and GRANT19 where Ruth had greater GNR (62.4 and 60.8 kg N ha^{-1}, respectively) than Freeman (53.2 and 56.8 kg N ha^{-1}, respectively). The main factor effect of N rate on GNR was significant at GRANT18, HPAL18, and HPAL19. The GNR ranged from 70.9 to 92.0, 31.4 to 56.1, and 44.2 to 53.3 kg N ha^{-1} at GRANT18, HPAL18, and HPAL19, respectively with the lowest GNR at zero N rate and the highest GNR at the highest N rate. At all three-site years, there was a linear increase in GNR with N rate.

A significant three-way effect of cultivar, N application timing, and N rate on NRE was observed at ENREC19 and HPAL19 (Table 6). Freeman with 25% N rate applied in Fall resulted in the greatest NRE at HPAL19 while Ruth with 25% N rate applied in Split had the greatest NRE at ENREC19. There were no obvious trends for differences in NRE by cultivar, N application timing, or N rate. There was a significant interaction effect of cultivar and N rate on NRE at PREC19. Ruth had significantly greater NRE (0.74 to 3.64 kgN kg^{-1} fertilizer N) than Freeman (0.59 to 3.09 kgN kg^{-1} fertilizer N) for each of the N rates except for the highest N rate (125%). There was an increase in NRE for each cultivar usually with a decrease in N rates. At ENREC18, Freeman had greater NRE compared to Ruth (0.12 > -0.07 kgN kg^{-1} fertilizer N). N rate had a significant main factor effect on NRE at GRANT19 where NRE for 25% N rate was significantly greater than other N rates and the increase in NRE was observed with a decrease in N rates.

Significant interaction effects of cultivar and N rate on PFP was observed at ENREC19 and HPAL18 (Table 6). Ruth had a higher PFP than Freeman at the lower N rates (0% and 25%) at ENREC19 and vice-versa for the control N treatment at HPAL18. There was no significant difference in PFP by cultivars at other N rates with one exception at HPAL18 where Freeman with 25% N rate had a significantly higher PFP than Ruth with 75% N rate.

There was a significant main factor effect of cultivar at two site-years. Ruth had a greater PFP than Freeman at PREC19 and the reverse was true at ENREC18. The main factor effect of N application timing on PFP was significant at GRANT18 where Split and Spring N application had greater PFP compared to Fall N application. There were significant main factor effects of N rate on PFP at five site-years which include both wet and dry site-years (ENREC18, GRANT18, GRANT19, HPAL19, and PREC19). At all of these site-years, zero N rate had the greatest PFP (11.37 to 67.62 kg grain yield kg^{-1} total available N) and PFP decreased with the increase in N rates.

Relationship among agronomic variables

A significant linear relationship was observed between grain protein content and grain yield at five site-years out of seven with slope coefficient ranging from -5.93 to 3.24 (g kg^{-1} grain protein content per Mg ha^{-1} grain yield) (Figure 9a). However, the fit of regression was low (R^2 = 0.03 to 0.35). Among the significant regressions, two site-years each had negative (GRANT19 and PREC19) and positive (HPAL18 and ENREC19) slopes. The regression at each site-year by N rate showed an inconsistent trend of slope coefficients across N rates (Supplementary Table 2). Analysis of variance on residuals of the above linear regression showed a significant main factor effect of N rate on GPD which suggested that grain protein content increased with increasing N rates when accounted for grain yield.

GNR increased significantly with the increase in grain yield at all site-years with slope coefficients ranging from 11.6 to 19.3 kg N Mg^{-1} grain yield (Figure 9e). Analysis of variance on residuals for the regression showed a significant main factor effect of cultivars and N rate on GNRD. Ruth had significantly higher GNR than Freeman (Figure 9f) and GNR increased with an increase in N rates (Figure 9g).

Post-harvest Residual Mineral Soil N

Residual total mineral N were in the order 125% N = 100% N > 75% N = 0% N at ENREC19 (Table 7). Residual mineral N was significantly greater with the 125% N than the control and 75% N at HPAL18. The N rate treatments at 125% and 100% had greater residual mineral N than the control at HPAL19. Residual mineral N did not vary by N rates at ENREC18, GRANT18, GRANT19, and PREC19. Overall, all sites in Year 2 tended to have greater residual mineral N than in Year 1.

DISCUSSION

Inter-annual differences in grain yield and grain protein

The experimental sites in this study covered a varied precipitation gradient and represent overall agroclimatic and production zones of winter wheat growing areas in Nebraska (Peterson, 1992). Greater grain yield in Year 1 than in Year 2 can be attributed to greater monthly and cumulative water supply compared to water demand during the growing season. Among all site-years with no disease pressure and hail damage, GRANT18 had a moderate cumulative WS:WD ratio (0.41) and distinctively higher ratio in March through

June (0.53 to 0.70) making ample water available during critical growth stages and thereby, had the highest of all grain yields (Deng et al., 2006; Bian et al., 2016). Mohammed et al. (2013) also reported similar results in a winter wheat experiment in Oklahoma, U.S. where grain yield was higher in years that received adequate precipitation during spring. These results support those by Partignani et al. (2014) in which as little as 250 mm growing season precipitation was enough to maximize winter wheat grain yield in Oklahoma, as long as precipitation distribution was favorable.

Severe infection of FHB caused yield loss at ENREC in Year 1 and therefore, there was no greater yield at ENREC in Year 1 than in Year 2 as was the case with other sites. For the same reason, ENREC did not yield the highest among the sites in Year 1 although it had a higher yield potential as it received more precipitation and a higher recommended N rate than other sites.

The decline in yield across site-years with a lower WS:WD ratio (0.18-0.21) compared to site-years with a greater WS:WD ratio (0.41-0.84) led to a higher grain protein content in drier site-years over wet site-years. Increased grain yield favored by adequate precipitation might have left minimal residual N in soil for crop uptake at the flowering stage and hence, lower grain protein content level in site-years with greater WS:WD ratio. Grain yield is reported to negatively correlate with grain protein content under low soil N condition and higher grain yield (Fowler, 2003; Kibite & Evans, 1984). This inverse grain yield-protein relation is attributed to the dilution of grain N resulted from a higher accumulation of carbohydrates in the kernel (Terman, 1979; Grant et al., 1985). Higher plant biomass from increased N uptake during early growth stages and low remobilization of N to the grains might also be a potential reason for inverse grain yield-protein relation (Gaju et al., 2014). However, the positive relationship between grain yield and protein observed at some site-years suggests that the negative relationship is not universal (Lollato et al., 2021; Torrion et al., 2019). Grain protein content is sometimes identified as an indicator of N sufficiency (Goos et al., 1982). The lower grain protein content (<12 g kg^{-1}) in site-years with greater WS:WD ratio (0.41-0.84) observed even with the high N rates suggest that additional N input could achieve greater yield and protein content in high-yielding environments.

Cultivar Differences

The linear regression of grain yield and grain protein content against environmental index suggested that both cultivars had broad adaptability for grain yield and grain protein content. Depending on annual precipitation, Ruth yielded higher in a dry year (3 out of 4 sites) and Freeman yielded higher in a wet year (2 out of 3 sites). Differences in yield between cultivars can be attributed to the cultivar characters as reported by Baenziger et al. (2020). Freeman is a cultivar developed for broad adaptation given temperature, elevation, and precipitation gradients present across the state and it yielded greater than Ruth under wet environment. Ruth as a semi-dwarf cultivar was developed more recently considering the moisture-limited environment and it yielded better than Freeman under dry environment. Greater PFP observed with Ruth in drier site-years and with Freeman in wet site-years also attest to similar advantage each cultivar has depending on growing environments. Otherwise, a statewide dryland wheat variety trial reported that grain yields averaged across years (2015-2017) were similar for these two cultivars in all the three wheat-growing regions in NE (southeast-includes ENREC, west central-includes GRANT, and west-includes HPAL and PREC) (NE Winter Wheat Variety Test, 2019). Late maturing cultivar will have a warmer grain filling condition and might accumulate more protein (Castro et al., 2007). Although Ruth has moderately late maturity compared to Freeman, there were no consistent cultivar differences in grain protein. In a given site-year where one cultivar had greater yield also had lower protein which rather suggested an inverse yield-protein relationship.

Nitrogen Application Time

Most of the N uptake by wheat occurs during stem elongation (Feekes GS 6 to 10) and N application prior to this stage has greater N loss potential (Zebarth et al., 2007). There was a substantial precipitation event (total of 48 mm) within 10 days after the fall application of fertilizer at GRANT18 (Figure 1). Precipitation is one of the important drivers of N loss from the root zone via nitrate leaching (Maharjan et al., 2014; Singh et al., 1995). Denitrification can also be a significant pathway for N loss in medium/heavy texture soils with water-logged conditions (Kaur et al., 2020). The possible loss of applied N might have resulted in a lower yield for fall N applied plots compared to split or spring treatments at GRANT18. At ENREC19, a similar increase in yield with spring or split N application was observed over fall application for Ruth. There was only around 2 mm precipitation within one week of fall N application at ENREC19. Therefore, this difference in yield by N application timing for Ruth suggests that this cultivar can potentially benefit from split or spring N application over fall application under rainfed condition even in a dry year given no or minimal disease pressure. Given the high variability in weather and grain yield response to applied N, split or spring application of N provides additional advantage allowing to make economy-based decision on whether to fertilize or terminate winter wheat as a cover crop in spring and plant other crops such as maize in spring.

Improvement in grain protein content of winter wheat with split applied N was reported in previous studies (Dhillon et al., 2020; Garrido-Lestache et al., 2004; Lollato et al., 2021). Our result aligned with the finding of Graham and Stockton (2019) who observed no significant increase in grain protein content by split N application over the at-sowing N application. Other studies reported an increase in grain protein content with fertilizer N top-dressed and foliar-applied in late spring (Zebarth et al., 2007; Weber et al., 2008; Mohammed et al., 2013). Dick et al. (2016) found that split N applied late-season during flag leaf stage (Feekes GS 9) and the post-flowering stage (Feekes GS 10.5.4) increased grain protein content in winter wheat in Oklahoma. In our experiment, the spring application of N was made at or around Feekes GS 5. Thus, this earlier application of spring dose of N fertilizer might have contributed to vegetative growth but not to increasing grain protein content in spring-applied N treatments.

Available N for crop uptake beyond vegetative stages or remobilization of plant accumulated N to grains would contribute to increasing grain protein content (Ottman et al., 2000; Vaughan et al., 1990; Doyle & Shapland, 1991). Therefore, it would require applying N rates greater than those considered in this study (particularly in spring of years with enough moisture) or applying additional N after the booting stage to observe an increase in grain protein content (Wang et al., 2008). Foliar application of N may be an effective strategy for in-season N management, when feasible, especially in dry conditions where N uptake from soil may be severely impacted (Wyatt et al., 2018; Dick et al., 2016; Woolfolk et al., 2002). However, one should account for a potential risk of leaf burn associated with late-season foliar N application, particularly at higher N rates and thereby, impacting grain yield (Cruppe et al., 2017). Urea with urease and nitrification inhibitor was reported to enhance winter wheat yield and protein in the U.S. Southern Plains (Adams et al., 2018). Advanced fertilizer technology such as polymer coating and chemical inhibitors slow N transformation in soil and thereby, may improve wheat yield and protein with a sustained supply of N during the season. In-season N management for greater protein should account for associated costs and returns (Corassa et al., 2018).

Nitrogen Rate

In a dryland environment such as Nebraska, grain yield and protein content can be co-limited by water and N availability (Sadras et al., 2016; Cossani & Sadras, 2018). Modeled indices of colimitation capturing water and N interaction affected wheat yield and nutrient use efficiency (Sadras, 2004). The site-years with a moderate WS:WD ratio (0.41 - 0.49) showed a significant increase in grain yield with an increase in N rates. These results aligned with the findings of Walsh et al. (2018) who reported an improved yield with increased fertilizer N application at experimental sites that had good soil moisture resulted from timely precipitation in a semi-arid environment. Availability of water during critical growth stages enhances N use efficiency in wheat (Ma et al., 2019; Ayad et al., 2010) and thereby, increases crop yield as N rate increases. When moisture is not limiting, an increase in available N to plants enhances dry matter accumulation by affecting leaf area, radiation interception, and photosynthetic efficiency (Srivastava et al., 2018; Duan et al., 2019). The positive linear response of grain yield with no plateau to N rates (including 125% of recommended N rate) observed at some site-years suggested that there is a potential to further increase grain yield by applying additional N when there is adequate soil moisture and low disease pressure.

The current NE winter wheat recommendation suggests for ≤ 112 kg N ha^{-1} for a maximum grain yield of 5 Mg ha^{-1} with $\geq$120 g kg^{-1} grain protein content under rainfed conditions (Hergert & Shaver, 2009). It does not directly account for the yield potential but rather, economics (cost of fertilizer and grain) of the production. As observed in the experiment, potential grain yield for a given site varied by year based on the weather conditions and the grain yield response to applied N also varied as a function of WS:WD. Therefore, a more robust fertilizer N recommendation can be developed with a consideration of yield potential that is co-limited by water and fertilizer. This can further open the opportunity for in-season N management using crop sensors that can determine crop N stress and need (Mullen et al., 2003; Raun et al., 2005) and crop water stress and needs (Sun et al., 2019). In addition to crop canopy reflectance data, a series of variables such as weather and soil moisture should be accounted for while developing a decision framework accompanied by machine learning techniques to improve the accuracy and reliability of in-season N rate recommendation (Colaco et al. 2021).

The decrease in yield with increasing N rate treatments at ENREC18 might be because of the effect of FHB on yield across all treatments. A similar result was reported by Varga et al. (2005) where no benefits in winter wheat grain yield was observed by increasing N rate under high disease severity and no fungicide application. Severity of FHB could be higher in wheat fertilized with higher N rates (Lemmens et al., 2004) and hence, a negative response of yield to N rate as was observed at ENREC18.

A drier soil environment in Year 2 due to lower precipitation during the growing season masked potential benefits of adding N at all sites (GRANT19, HPAL19, and PREC19) except for ENREC19 as evidenced by higher residual mineral N particularly under high N rate treatments in Year 2 than in Year 1. Greater yield with increasing rates of N was observed at ENREC19 because yield potential was considerably higher there in Year 2 compared to other sites. Although Year 2 was a drier year than normal, ENREC19 was not too dry nor as drastically dry as other three sites. At PREC19, there was also a high pre-plant residual nitrate-N (220 kg ha^{-1}) at 0-120 cm depth and such high residual soil N reduces fertilizer N use efficiency (Matson et al., 1998; Duan et al., 2019) and makes N application less effective to increase yield, particularly in a drier environment.

Increase in grain protein content with N rate observed across site-years in this experiment align with the findings of Mohammed et al. (2013) in an experiment conducted in Oklahoma, U.S., where they reported an increase of grain protein content with increasing fertilizer N in winter wheat while the increment in protein content was variable across years. There are other studies that reported similar results (Zhang et al., 2017; Bhatta et al., 2017; Lollato et al., 2019). However, Abedi et al., (2011) reported that over-application of fertilizer N (360 kg N ha^{-1}) can result in a drop in protein content in wheat grains due to dilution effect from increased grain yield.

Other agronomic responses (GNR, NRE, and PFP)

The average NRE observed across site-years (0.02 – 0.25 kgN kg^{-1} fertilizer N, excluding PREC19) was lower compared to the estimated global NUE of 0.35 kgN kg^{-1} for cereal crops (Omara et al., 2019) and those reported for the U.S. Great Plains region (Lollato et al., 2021). The exceptionally higher NRE observed at PREC19 (1.52 kgN kg^{-1} fertilizer N) could be attributed to the higher pre-plant residual NO_3-N (Yan et al., 2014), which increased available N for uptake while it was not accounted for in NRE calculation. A decrease in PFP with the increase in N rates, as observed in the study, has been widely reported (Lollato et al., 2021; Yang et al., 2019) and can be attributed to inefficient crop N uptake or loss of N via different pathways.

CONCLUSION

Nitrogen fertilization increased grain yield only in wet environments while it increased grain protein content in both dry and wet environments. Spring and split applied N can be effective over fall application in those years when there is a greater risk of applied N loss via leaching and denitrification. Ruth, as a semi-dwarf most recently developed cultivar for greater adaptation in NE and neighboring states demonstrated potential to benefit from spring or split N application given no disease pressure. An inverse relationship between grain yield and protein was prominent in dry environments while there was a positive relationship in wet and disease-free environment. Available soil N for crop uptake beyond the vegetative stage is critical to increasing grain protein content. Further research on applying fertilizer N beyond Feekes GS 10 or the use of available fertilizer technologies such as controlled- or slow-release N to achieve prolonged and sustained soil N through the flowering stage is warranted. An effective N management strategy for winter wheat should account for and be adaptable to weather variability, particularly available moisture during spring to optimize grain yield and protein content in winter wheat. This may be achieved using in-season N management tools like crop canopy sensors, the potential of which needs to be further explored in dryland winter wheat. Variability in weather and its impact on yield potential observed in this experiment indicated that directly accounting for yield potential into the N recommendation algorithm, which is missing in the current algorithm, can be useful for successful N management. Future studies on the economics of the cost associated with differential N fertilization and the returns from yield gains and protein premiums can aid winter wheat growers in making appropriate decisions. With the premium being paid for higher proteins, it might be worth an effort to incorporate grain protein component directly into the N recommendation as attempts are made to revise and improve current algorithms.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Deepak Ghimire: Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Writing-original draft, Writing-review & editing. **Saurav Das:** Data curation, Formal analysis, Writing-original draft, Writing-review & editing. **Cody Creech**: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing-original draft, Writing-review & editing. **Dipak Santra:** Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing-original draft, Writing-review & editing. **Nathan Mueller**: Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing-review & editing. **Brian Maust**: Investigation, Methodology, Project administration, Resources, Writing-review & editing. **Amanda Easterly**: Data curation, Investigation, Methodology, Project administration, Resources, Writing-review & editing. **P. Stephen Baenziger**: Conceptualization, Funding acquisition, Investigation, Methodology, Writing-review & editing. **Bijesh Maharjan**: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing-original draft, Writing-review & editing.

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FIGURE 1. Cummulative precipitation and ET_o; reference evapotranspiration (mm) at different site-years across Nebraska during winter wheat growing seasons. The arrows shows the dates for sowing (P), fall fertilization (F), spring fertilization (S), and harvest (H). The ratio of water supply and demand during the season (WS:WD) are given for each site-year.

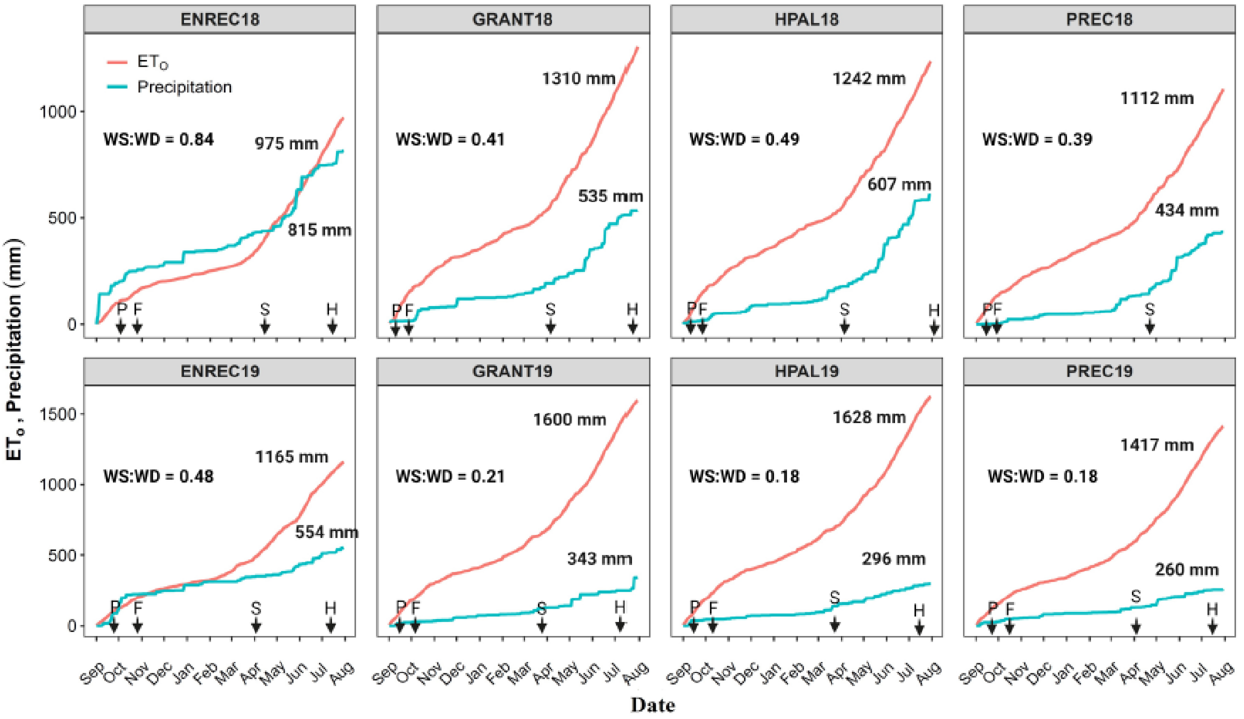

FIGURE 2. Linear regression of a) Grain yield and b) Grain Protein content for different cultivars against environmental index.

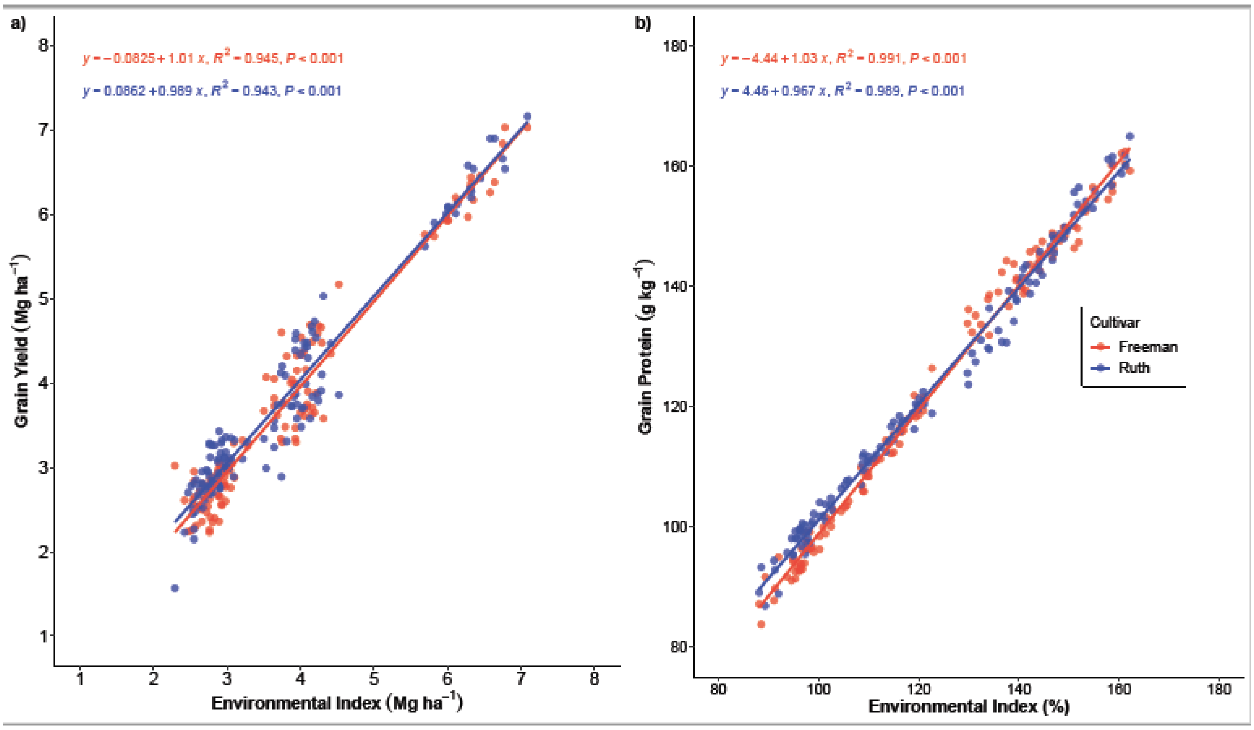

FIGURE 3. Regression of winter wheat a) grain yield and b) grain protein content for different site-years against water supply and water demand ratio (WS:WD). Grain yield data from GRANT18 was excluded from regression considering it as an outlier in Figure 3a.

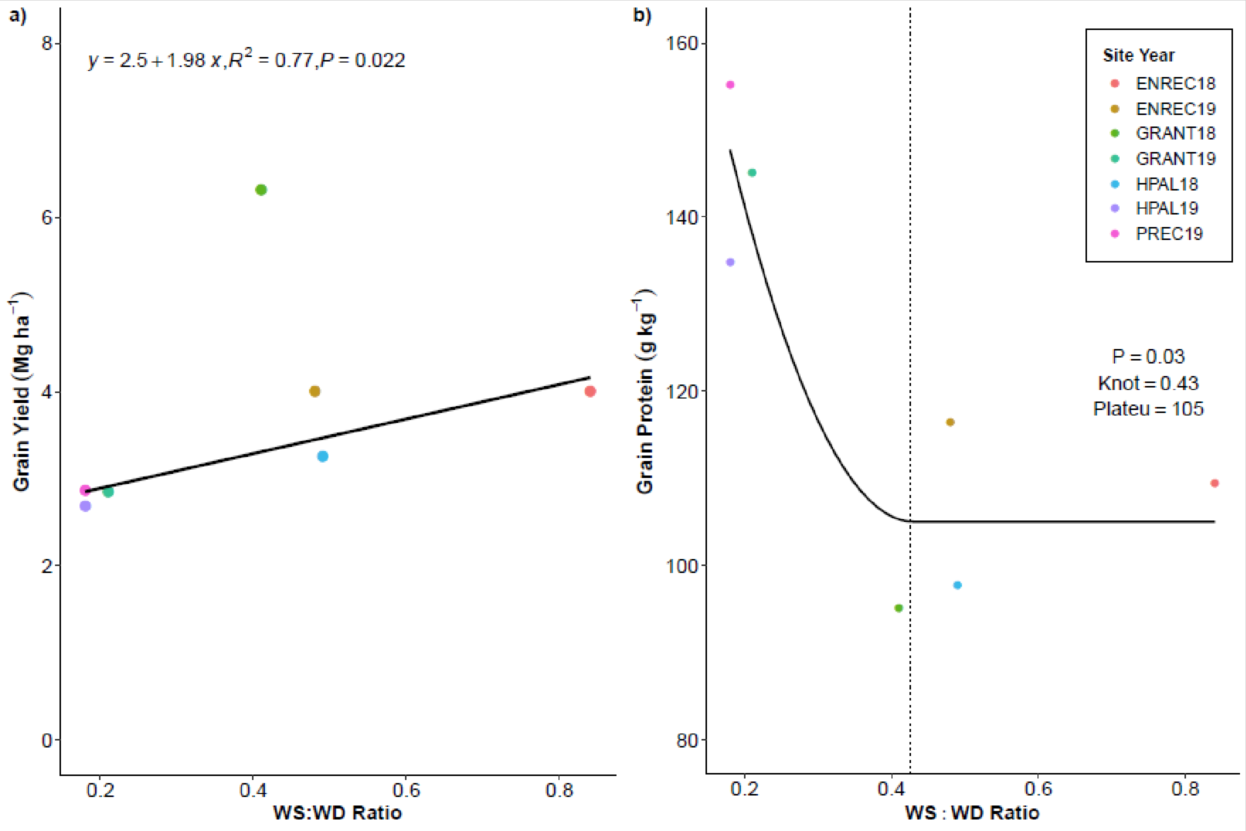

FIGURE 4. Linear regression (with regression coeffient and *P*-value) of winter wheat grain yield against available N (kg ha^{-1}) at different experimental sites across Nebraska during winter wheat growing season in a) Year 1 (2018/2019) and b) Year 2 (2019/2020).

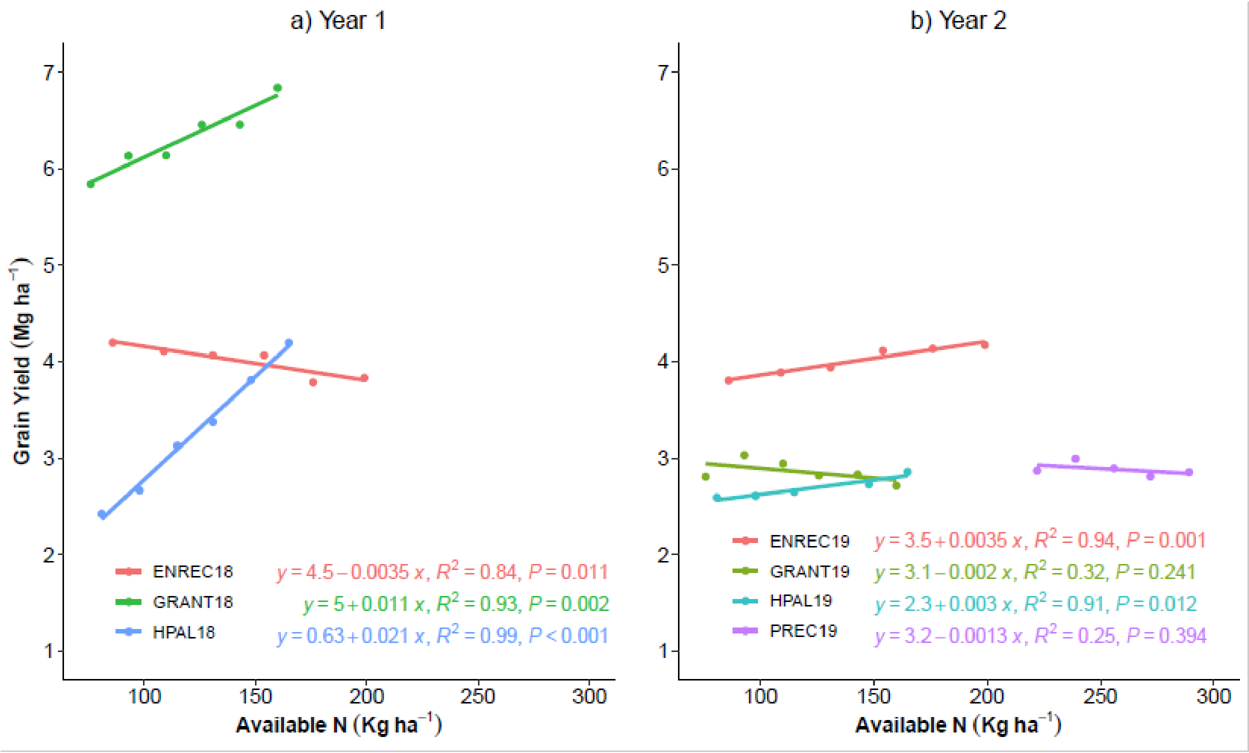

FIGURE 5. Winter wheat grain yield as affected by a) Interaction of cultivar and N rate at ENREC19 (2019/2020); b) Interaction of cultivar and N rate at PREC19 (2019/2020); and c) Interaction of cultivar and N application time at ENREC19 (2019/2020). Bars with different uppercase letters indicate significant difference in mean grain yield at *P* <0.05.

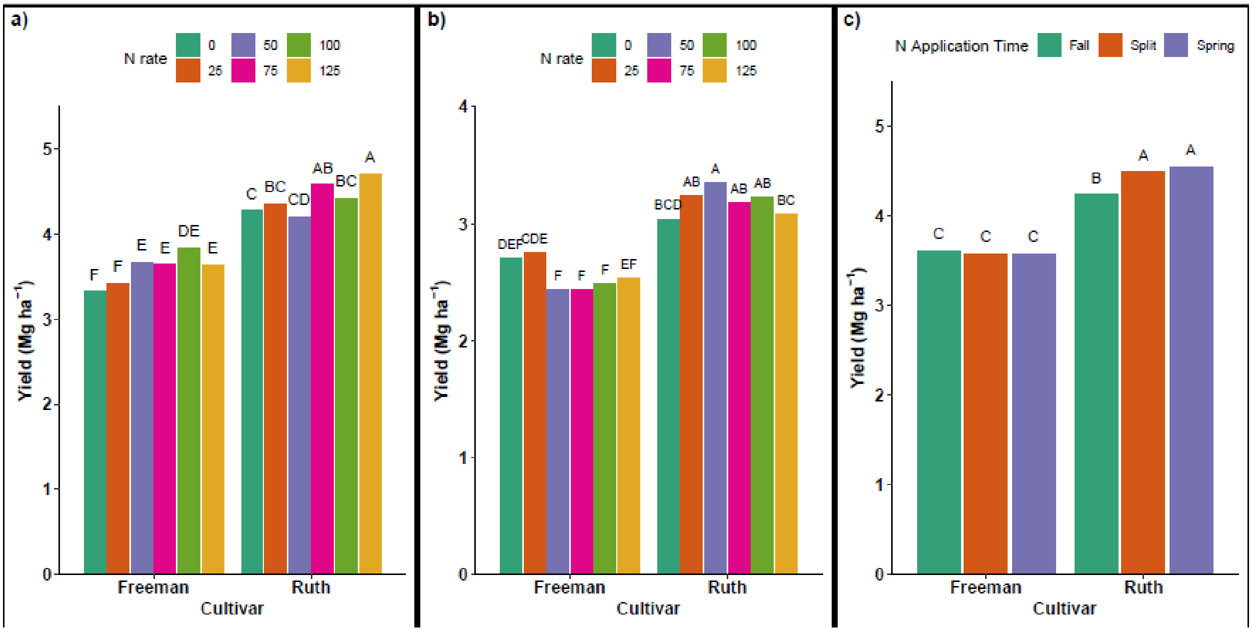

FIGURE 6. Linear regression (with regression coeffient and *P*-value) of winter wheat grain protein against available N (kg ha^{-1}) across different experimental sites across Nebraska during winter wheat growing seasons in a) Year 1 (2018/2019) and b) Year 2 (2019/2020).

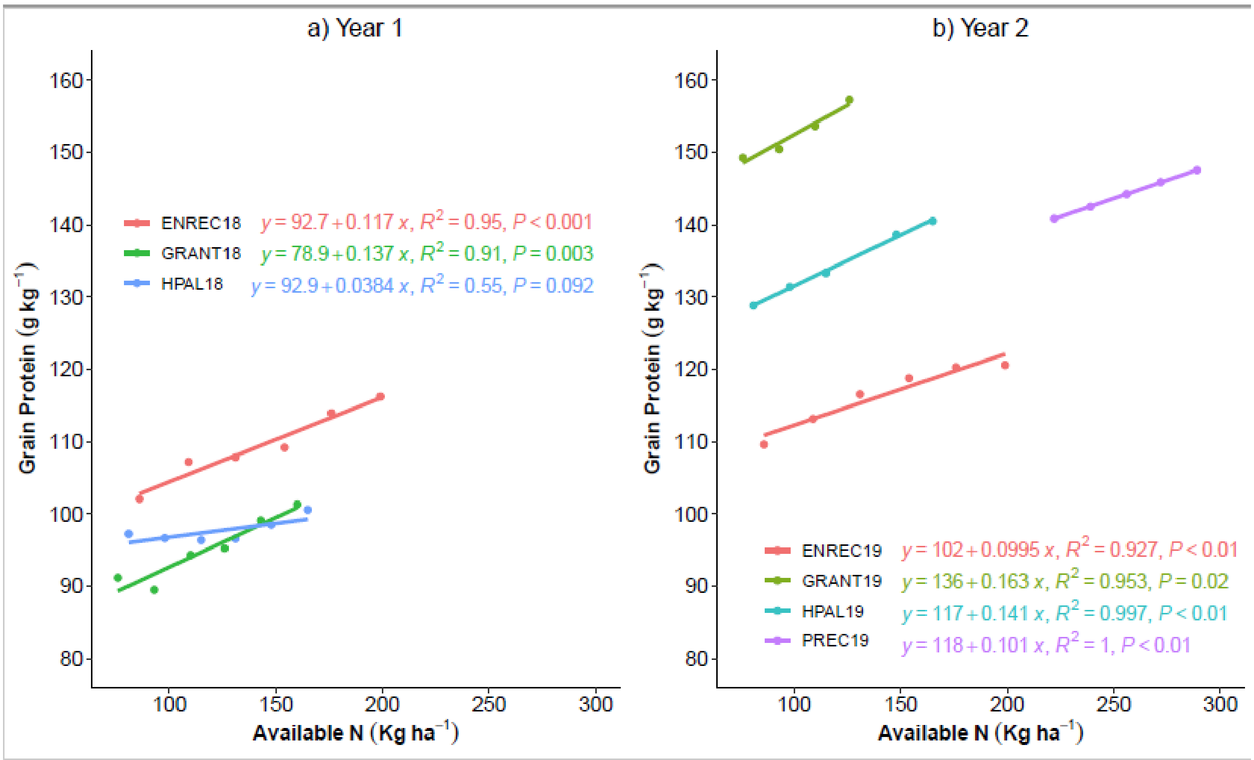

FIGURE 7. Grain protein of winter wheat as affected by the interaction of cultivar and N rate at HPAL18 (2018/2019). Bars with different uppercase letters indicate significant difference in mean grain protein at *P* <0.05.

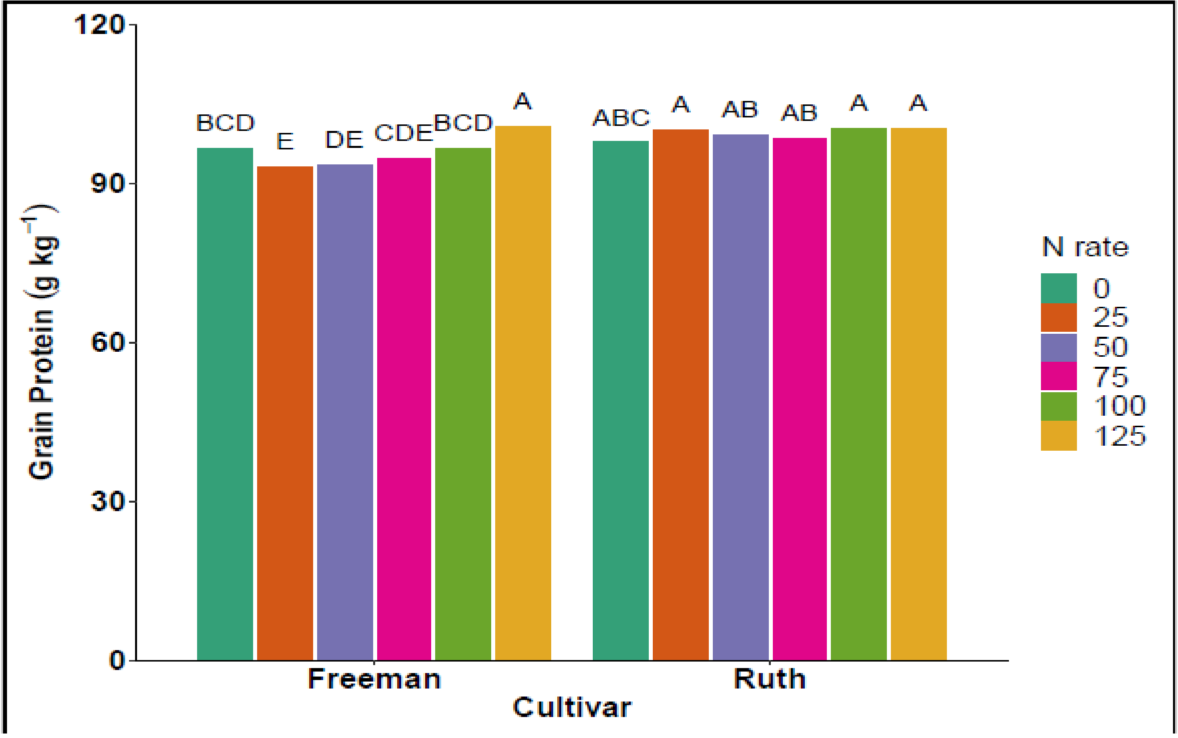

FIGURE 8. Grain yield of winter wheat as affected by the interaction of N application time and N rate at HPAL19 (2019/2020). Bars with different uppercase letters indicate significant difference in mean grain protein at *P* <0.05.

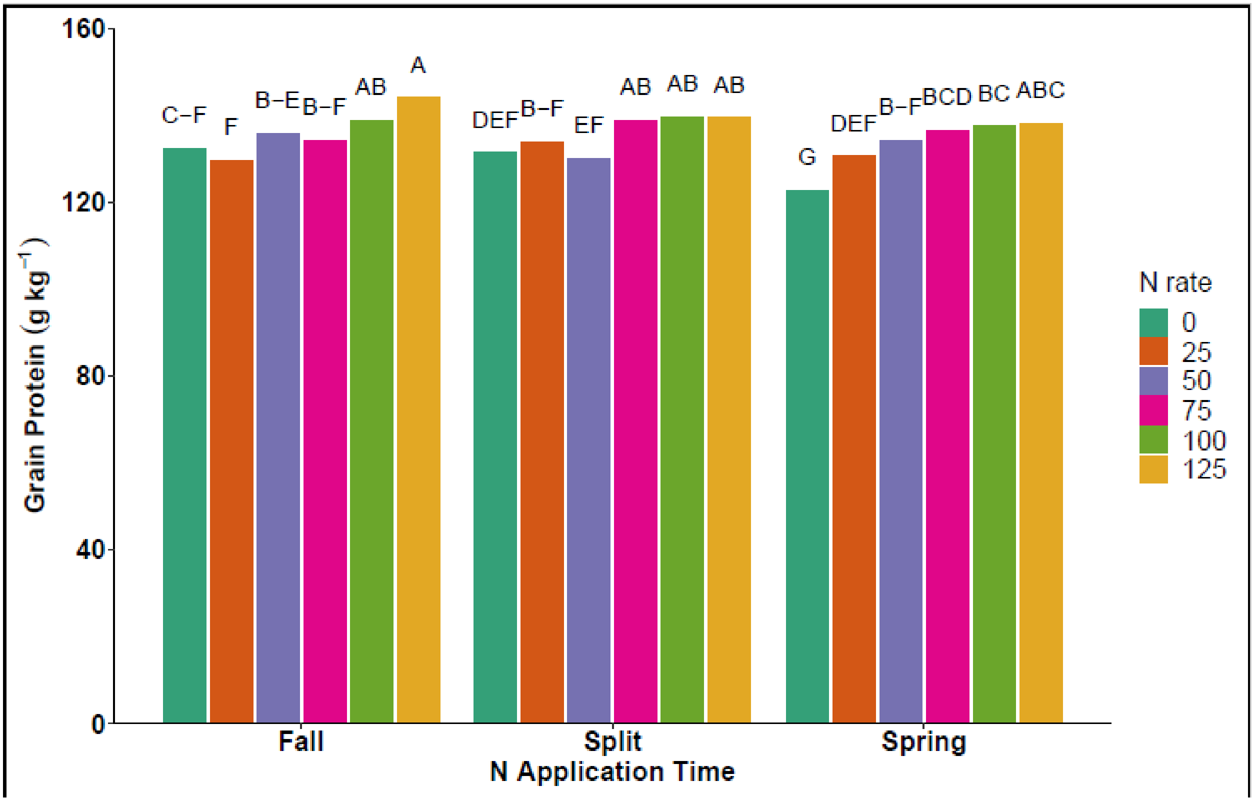

FIGURE 9. Relationship between grain yield and grain protein content (a), and between grain yield and grain N removal (e). Analysis of variance for the residuals of the regressions in (a) and (e) are shown for N rate (b, f), cultivar (c, g), and N application timing (d, h). Error bars represent standard error.

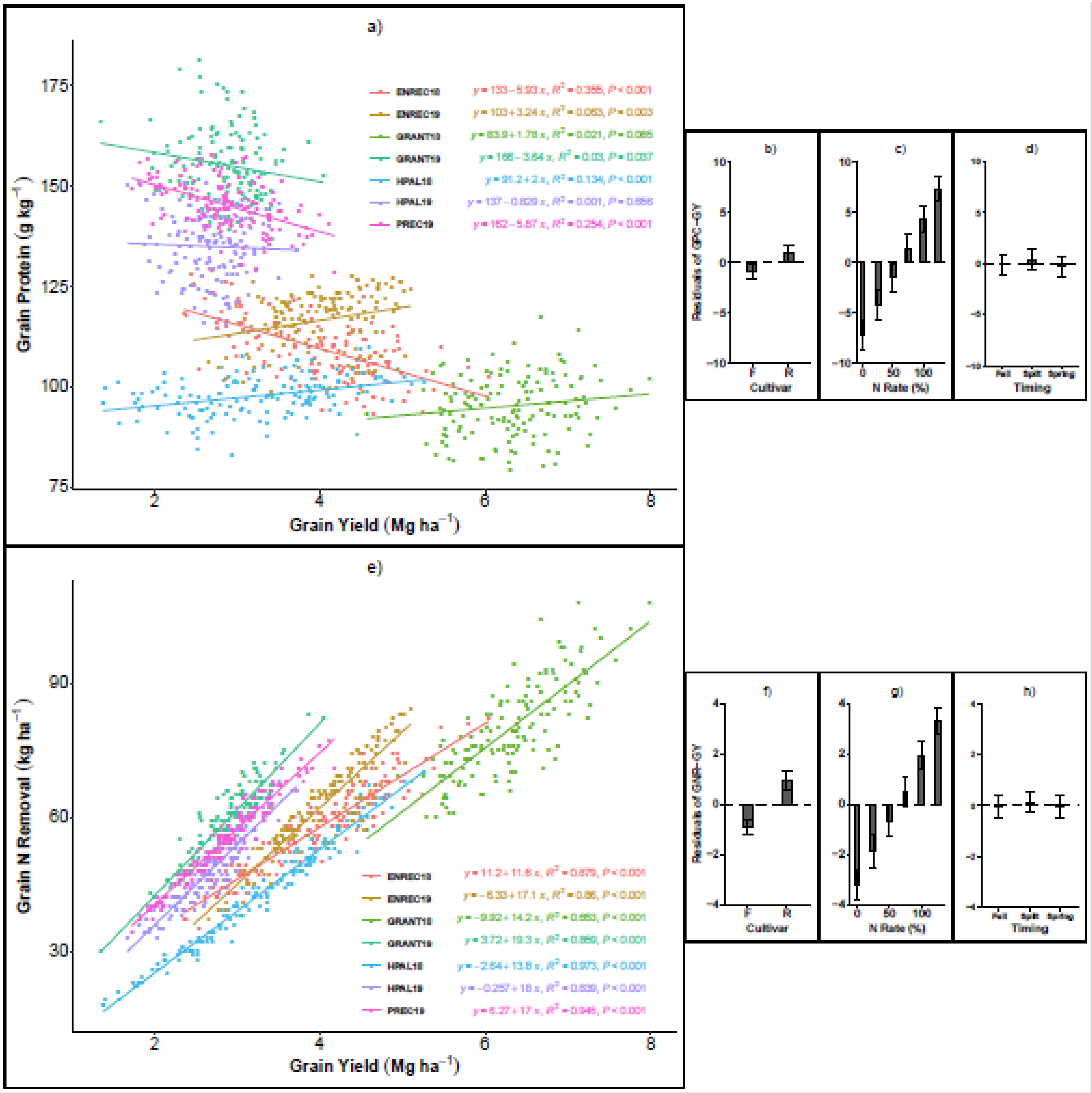

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TABLE 1. Chemical properties of soil collected pre-sowing at different site-years across Nebraska during winter wheat growing seasons in Year 1 (2018/2019) and Year 2 (2019/2020).

Site-year	**Nitrate-N**			**Olsen P**	**K**	**Organic Matter**	**Soil pH**
	0-20 cm	*20-60 cm*	*60-120 cm*	——— *0-20 cm* ———			
	——— kg ha^{-1} ———			——— mg kg^{-1} ———			
ENREC18	19.1	28.7	37.2	10.9	268	36	6.7
GRANT18	36.6	16.3	22.4	18.8	427	23	7.2
HPAL18	26.1	23.4	30.5	21.1	647	28	7.8

PREC18	3.1	23.9	35.8	3.6	465	18	8.5
ENREC19	1.2	2.4	2.5	11.9	287	36	7.3
GRANT19	24.8	20.3	23.3	29.4	260	13	6.5
HPAL19	20.5	22.1	23.3	9.7	743	29	7.3
PREC19	42.5	126	52.9	8	545	20	8

TABLE 2. Pairwise comparison of response variables by cultivars in the control (zero N) treatment at seven site-years

Site-Year	Grain yield (Mg ha^{-1})		Grain Protein (g kg^{-1})		Grain Protein Deviation		Grain N Removal (kg N ha^{-1})		Partial Factor Productivity (kg grain kg^{-1} N)	
	F□	R	F	R	F	R	F	R	F	R
ENREC18	4.47^{a}□	3.92^{b}	99.9^{b}	104.3^{a}	-14.3	-15.5	59.15	54.06	45.71^{a}	40.13^{b}
ENREC19	3.33^{b}	4.28^{a}	107.8	111.4	-18.2^{b}	-4.7^{a}	47.89^{b}	63.33^{a}	487.79^{b}	627.73^{a}
GRANT18	5.81	5.87	88.4^{b}	93.9^{a}	-11.7	-5.6	68.29	73.44	67.28	67.96
GRANT19	2.73	2.88	147.1	151.4	14.8	20.6	53.38	57.80	34.86	36.71
HPAL18	2.86^{a}	1.98^{b}	96.7	97.7	-34.2	-42.4	37.03^{a}	25.74^{b}	31.06^{a}	21.54^{b}
HPAL19	2.54	2.64	131.8^{a}	125.8^{b}	-2.5	-7.4	44.29	44.16	33.86	35.21
PREC1	2.70^{b}	3.04^{a}	139	142.	7.1^{b}	12.9^{a}	49.9	57.1	10.70^{b}	12.04^{a}

9		.7 0		0^{b} 4^{a}	

□ F and R represent Freeman and Ruth cultivars.

□ Mean values followed by different lowercase letters in each variable column indicate significant difference at $P<0.05$ for each site-year

TABLE 3. Mean grain yield and grain protein of winter wheat affected by site-year, cultivar, time of nitrogen application and nitrogen rate during winter wheat growing seasons in Year 1 (2018/2019) and Year 2 (2019/2020) across Nebraska.

Source of Variation	**Yield**	**Protein**
	— Mg ha^{-1} —	—— g kg^{-1} ——
Site-year (SY)		
ENREC18	4.0^{b}†	109.4^{e}
GRANT18	6.3^{a}	95.1^{g}
HPAL18	3.3^{c}	97.7^{f}
ENREC19	4.0^{b}	116.5^{d}
GRANT19	2.9^{d}	155.2^{a}
HPAL19	2.7^{d}	134.9^{c}
PREC19	2.9^{d}	145.2^{b}
P-value	**<.0001**	**<.0001**
Cultivar (C)		
Ruth	3.9	119.9
Freeman	3.8	118.8
P-value	0.0670	0.1193
Time of N Application (T)		
Split	3.8	119.5
Fall	3.8	119.2
Spring	3.8	119.3
P-value	0.5874	0.8157
N Rate (R)□		
0	3.6^{d}	114.5^{f}
25	3.7^{c}	116.1^{e}
50	3.8^{bc}	118.2^{d}
75	3.9^{b}	120.0^{c}
100	3.9^{ab}	122.7^{b}
125	4.0^{a}	124.5^{a}
P-value	**<.0001**	**<.0001**
	—————— ***P* – value** ——————	
Interactions		
SY x C	**<.0001***	**<.0001**
SY x T	0.2521	0.0707
SY x R	**<.0001**	**0.0109**

C x T	0.1527	0.4413
C x R	0.6763	0.5507
T x R	0.8830	0.9560
SY x C x T	0.8831	0.9978
SY x C x R	0.5893	0.6402
SY x T x R	0.7898	0.7089
C x T x R	0.7605	0.7865
SY x C x T x R	0.7869	0.8907

†Mean values followed by different letter in each column and section indicate significant difference at a given *P* value.

**P* value lower than 0.05 is in bold fonts.

□Unit of N rate is % of recommended N rate

TABLE 4. Mean grain yield of winter wheat for each site-year as affected by cultivar, nitrogen application time, and nitrogen application rate.

Source of Variation	**Grain Yield**						
	ENREC18	**GRANT 18**	**HPAL 18**	**ENREC 19**	**GRANT 19**	**HPAL 19**	**PREC 19**
				Mg ha^{-1}			
Cultivar (C)							
Ruth	3.64^{b}†	6.35	3.07^{b}	4.43^{a}	2.94^{a}	2.71	3.18^{a}
Freeman	4.37^{a}	6.27	3.46^{a}	3.59^{b}	2.77^{b}	2.68	2.56^{b}
P- value	**<.0001**	0.76	**0.032**	**0.010**	**0.009**	0.660	**0.009**
Time of N Application (T)							
Split	4.06	6.38^{a}	3.33	4.04	2.86	2.69	2.81
Fall	4.04	6.16^{b}	3.31	3.93	2.85	2.73	2.94
Spring	3.93	6.39^{a}	3.15	4.06	2.86	2.67	2.86
P- value	0.558	**0.019**	0.268	0.056	0.992	0.713	0.156
N Rate (R)□							
0	4.19	5.84^{d}	2.42^{d}	3.80^{b}	2.81	2.59	2.87

25	4.11	6.13^{c}	2.66^{d}	3.86^{b}	3.03	2.61	2.99
50	4.06	6.14^{c}	3.13^{c}	3.94^{b}	2.94	2.65	2.89
75	4.06	6.46^{b}	3.37^{c}	4.11^{a}	2.82	2.74	2.81
100	3.83	6.46^{b}	3.81^{b}	4.14^{a}	2.83	2.73	2.85
125	3.78	6.84^{a}	4.19^{a}	4.19^{a}	2.71	2.86	2.81
P – value	0.2048	**<.0001**	**<.0001**	**<.0001**	0.093	0.097	0.391
				***P* – value**			
Interactions							
C x T	0.5112	0.714	0.795	**0.008***	0.954	0.122	0.186
C x R	0.868	0.409	0.211	**0.004**	0.914	0.635	**0.044**
T x R	0.561	0.557	0.647	0.108	0.325	0.193	0.409
C x T x R	0.350	0.396	0.765	0.273	0.497	0.262	0.409

† Mean values followed by different letter in each column and section indicate significant difference at a given *P* value.

**P* value lower than 0.05 is in bold fonts.

□Unit of N rate is % of recommended N rate

TABLE 5. Mean grain protein content of winter wheat for each site-year affected by cultivar, nitrogen application time, and nitrogen application rate.

Source of Variation	Grain Protein						
	ENREC 18	GRANT 18	HPAL 18	ENREC 19	GRANT 19	HPAL 19	PREC 19
				g kg^{-1}			
Cultivar (C)							
Ruth	110.7	96.6	99.4^{a}	117.0	155.9	132.0^{b}	145.3

Freeman	108.1	93.6	95.9^{b}	115.9	154.5	137.7^{a}	145.1
P–value	0.21	0.20	**0.02**	0.82	0.40	**0.01**	0.91
Time of N Application (T)							
Split	109.2	95.1	98.3	116.7	153.9	135.5	146.4
Fall	108.1	94.6	97.5	116.1	156.9	135.8	144.9
Spring	110.9	95.6	97.2	116.7	154.8	133.2	144.3
P-value	0.07	0.69	0.36	0.53	0.15	0.08	0.11
N Rate (R)□							
0	102.1^{c}†	91.2^{cd}	97.2^{b}	109.6^{e}	149.2^{d}	128.8^{e}	140.8^{e}
25	107.2^{b}	89.5^{d}	96.7^{b}	113.0^{d}	150.4^{cd}	131.4^{de}	142.5^{de}
50	107.8^{b}	94.3^{bc}	96.4^{b}	116.5^{c}	153.6^{bc}	133.3^{cd}	144.2^{cd}
75	109.2^{b}	95.2^{b}	96.6^{b}	118.8^{b}	157.3^{ab}	136.6^{bc}	145.9^{bc}
100	113.9^{a}	99.1^{a}	98.5^{ab}	120.3^{ab}	160.1^{a}	138.6^{ab}	147.5^{ab}
125	116.2^{a}	101.3^{a}	100.6^{a}	120.7^{a}	160.6^{a}	140.5^{a}	150.3^{a}
P-value	**<.0001**	**<.0001**	**0.0039**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
				***P*–value**			
Interactions							
C x T	0.9915	0.6937	0.4057	0.6558	0.9233	0.4802	0.4672
C x R	0.9565	0.5510	**0.0236** *	0.0771	0.3909	0.2482	0.2972
T x R	0.4554	0.4055	0.3827	0.4626	0.8872	**0.0380**	0.6026
Cx T x R	0.7533	0.2554	0.8098	0.1566	0.7591	0.1677	0.4572

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† Mean values followed by different letter in each column and factor indicate significant difference at a given *P* value using least-squares means.

**P* value lower than 0.05 is in bold fonts.

□Unit of N rate is % of recommended N rate

TABLE 6. Three-way ANOVA F-test probabilities for Grain Nitrogen Removal (GNR), Nitrogen Recovery Efficiency (NRE), and Partial Factor Productivity (PFP) as affected by cultivar (C), N rate, N application timing(Timing), and their interaction at seven site-years in Nebraska.

	C	**Timing**	**Nrate**	**C*Timing**	**C*Nrate**	**Timing*Nrate**	**C*Timing*Nrate**
GNR							
ENREC18	**<.0001**	0.72	0.91	0.37	0.54	0.75	0.39
ENREC19	**0.02**	**0.04**	**<.0001**	**0.01**	**0.02**	0.13	0.47
GRANT18	0.27	**0.01**	**<.0001**	0.52	0.73	0.26	0.55
GRANT19	**0.0029**	0.88	0.30	0.89	0.99	0.22	0.46
HPAL18	0.10	0.30	**<.0001**	0.70	0.22	0.85	0.80
HPAL19	0.14	0.28	**<.0001**	0.12	0.93	0.21	0.13
PREC19	**0.004**	0.11	0.40	0.24	**0.03**	0.18	0.33
NRE							
ENREC18	**0.02**	0.29	0.95	0.29	0.11	0.26	0.63
ENREC19	**0.03**	**0.009**	0.94	**0.007**	**<.0001**	0.11	**0.02**
GRANT18	0.46	0.07	0.13	0.96	0.74	0.05	0.56
GRANT19	0.15	0.93	**0.006**	0.47	0.91	0.95	0.26
HPAL18	0.47	0.39	0.58	0.66	0.86	0.94	0.98
HPAL19	0.26	0.32	0.94	**0.01**	0.99	0.32	**0.02**
PREC19	**<.0001**	0.07	**<.0001**	0.70	**0.004**	0.80	0.94
PFP							
ENREC18	**<.0001**	0.53	**<.0001**	0.80	0.80	0.36	0.49
ENREC19	**0.010**	0.40	**<.0001**	0.33	**<.0001**	0.84	0.91
GRANT18	0.80	**0.03**	**<.0001**	0.81	0.55	0.66	0.68

GRANT19	0.09	0.93	**<.0001**	0.93	0.95	0.32	0.49
HPAL18	**0.049**	0.51	**0.049**	0.67	**0.02**	0.78	0.65
HPAL19	0.50	0.80	**<.0001**	0.13	0.82	0.44	0.46
PREC19	**0.01**	0.24	**<.0001**	0.23	0.08	0.32	0.53

TABLE 7. Residual total mineral (nitrate + ammonium) nitrogen in postharvest soil samples (0-90 cm) under different nitrogen rates at all site-years across Nebraska during winter wheat growing seasons in Year 1 (2018/2019) and Year 2 (2019/2020).

N rate[□]	**ENREC18**	**ENREC19**	**GRANT18**	**GRANT19**	**HPAL18**	**HPAL19**	**PREC19**
	——— kg N ha^{-1} ———						
0	28.0	53.1^{b}†	45.8	131.1	19.2^{b}	43.9^{b}	219.8
75	34.9	75.4^{b}	49.8	155.0	17.3^{b}	51.1^{ab}	230.0
100	33.5	110.0^{a}	51.5	100.2	20.1^{ab}	67.7^{a}	261.0
125	32.2	101.7^{a}	53.9	112.8	26.5^{a}	63.6^{a}	294.7
P- value	0.51	**<0.0001**	0.69	0.14	**0.042**	**0.028**	0.52

[†] Means followed by same letter in each column are not significantly different at $p < 0.05$ using least-squares means.

[□] Unit of N rate is % of recommended N rate

Supplementary Table 1. Actual rates of N fertilizer applied for different treatments at each experimental location.

Treatments	ENREC	GRANT	HPAL	PREC
	N Rate, kg ha^{-1}			
25%	23	17	17	17
50%	45	34	34	34
75%	68	50	50	50
100%	90	67	67	67
125%	113	84	84	84

Supplementary Table 2. Slope coefficients ± standard error of regression between grain protein content and grain yield at each site-year dissected by N rates.

N Rate	ENREC18	ENREC19	GRANT18	GRANT19	HPAL18	HPAL19	PREC19
0	**-5.99 ± 1.72**	**5.03 ± 2.21**	3.92 ± 2.59	-1.51 ± 5.4	1.21 ± 0.91	-4.57 ± 4.39	**-6.18 ± 1.9**
25	**-4.67 ± 1.4**	3.05 ± 2.35	-4.64 ± 2.76	-1.49 ± 3.11	0.83 ± 1.68	-2.91 ± 4.9	**-7.43 ± 2.03**
50	**-5.6 ± 1.01**	-0.08 ± 2.29	**-7.39 ± 2.42**	1.31 ± 2.46	4.34 ± 1.67	**-0.93 ± 3.95**	**-5.49 ± 1.44**
75	**-3.57 ± 1.39**	-0.79 ± 2.38	-2.39 ± 2.34	2.6 ± 5.04	3.09 ± 1.23	**-2.88 ± 3.9**	**-6.28 ± 1.41**
100	**-7.3 ± 1.39**	2.73 ± 3.18	-1.95 ± 2.05	-7.74 ± 4.3	1.46 ± 1.97	-6.68 ± 3.58	-3.25 ± 1.69
125	**-3.59 ± 1.18**	0.14 ± 1.94	-0.29 ± 2.66	-2.48 ± 3.02	1.79 ± 1.67	-2.24 ± 4.3	-4.58 ± 2.43

□Bold values are significant at $P<0.05$