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RESEARCH ARTICLE

Impact of in-season split application of nitrogen on intra-panicle grain dynamics, grain quality, and vegetative indices that govern nitrogen use efficiency in sorghum

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Abstract

Background: The correct rate and timing of nitrogen (N) has the potential to improve sorghum productivity through modified grain yield components and quality. The impacts of in-season split application of N have little documentation.

Aim: An experiment was conducted to determine the optimum rate and timing of N to relate vegetative indices that govern nitrogen use efficiency and to maximize grain yield and quality under different soil types.

Methods: Pioneer 86P20 was grown in three environments on two different soil types following a completely randomized block design with nine N application treatments. Treatments included differing N rates applied at critical developmental stages of sorghum (planting, panicle initiation, and booting), accompanied with high temporal aerial phenotyping.

Results: Opportunities to increase grain protein content while using split N applications were observed, with panicle initiation identified as a critical developmental stage. In-season split application of N enhances grain yield under low soil mineral N. Split application of 31 kg N ha^{-1} each at the time of planting, panicle initiation, and booting emerged as optimum N treatment to increase protein content in sorghum. Vegetative indices, that is, normalized difference vegetation index and normalized difference red edge index are capable of predicting grain yield and protein content, respectively. Intra-panicle grain numbers and weights were altered significantly at different portions within panicles, with an opportunity to enhance yield potential at the bottom portion. The strong stay-green trait in this hybrid locked a large proportion of nitrogen in the leaves, which warrants the need for balancing stay-green and senescence in sorghum improvement programs.

Conclusions: Findings highlight that in grain sorghum remobilization of residual leaf N into grain is a target to increase yield and grain quality. An optimized stay-green trait balanced with senescence is recommended for enhancing sorghum yield potential.

KEYWORDS
grain protein, intra-panicle grain filling, nitrogen use efficiency, sorghum, source–sink dynamics

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1 | INTRODUCTION

Grain sorghum [*Sorghum bicolor* (L.) Moench] is a semiarid crop that is well adapted to heat and drought stress conditions (Blum, 1970; Chiluwal et al., 2018; Doggett, 1988; Pennisi, 2009; Sunoj et al., 2017). Further, sorghum is known to be productive under marginal soils with low nutrient supply (Qi et al., 2016) and sub-optimal environments (Saballos, 2008; van Oosterom et al., 2001). These adaptations have propelled sorghum into a major agricultural commodity that has a significant impact on the economy of the Great Plains of the US, Asian, and African countries (Hariprasanna & Rakshit, 2016; Leff et al., 2004; Nagaraj et al., 2013). Over the last 5 years (2018–2022), on average Kansas and Texas had 1238,300 and 688,000 ha of sorghum, which accounted for 50.2% and 28.0% of the total hectares of sorghum grown in the United States, respectively. Sorghum grain yield average for the United States over the past 5 years is 4.12 t ha^{-1}, whereas it is 4.71 and 3.57 t ha^{-1} for Kansas and Texas, respectively (https://www.nass.usda.gov).

Although sorghum has low nutrient requirements to be productive, greater yields can be achieved with adequate nitrogen (N) supply (Bollam et al., 2021; Mahama et al., 2014). Nitrogen is the most limiting nutrient in modern crop production (Hirel et al., 2011), as it plays a critical role in determining yield, protein, and amino acid composition in the grain (Bollam et al., 2021; Mahama et al., 2014; Waggle et al., 1967). In addition, N is a key component in plant pigments, including chlorophyll, which increases photosynthesis and translates to higher growth rate and yield (Dovale et al., 2012; Muchow & Sinclair 1994). The total amount of N required by sorghum is dependent on many factors, such as soil–plant available water content, available soil N, organic matter, tillage practice, preceding crop, and planting density (Halvorson et al., 2002; Rego et al., 1988; Wortmann et al., 2007). As most soils lack the adequate supply of N required for the optimal growth of sorghum, an external application of N fertilizer is a common practice. Globally, only 47% of N from applied fertilizers on cropland is being recovered, indicating that more than 50% of N applied as fertilizer to crops is lost to the environment (Lassaletta et al., 2014). Similarly, Omara et al. (2019) found that the NUE of cereal crops, including sorghum, remained unchanged over the past decade. A study on NUE across irrigations found that sorghum had an NUE of 35%–40%, and up to 19% of yield losses in sorghum are attributed to nutrient deficiencies, mainly N (Sigua et al., 2018).

Several studies have investigated the impact of the rate and timing of N fertilization on grain yield in sorghum. A linear increase in sorghum grain yield was noticed with increase in the amount of N applied from 0 to 87 kg N ha^{-1} and a split application at planting and panicle initiation enhanced grain yield compared to N applied only at planting in the Ethiopian Highlands (Melaku et al., 2018). In another study in Kansas, sorghum under three different N rates recorded maximum grain yield at 90 kg N ha^{-1} and hybrids outperformed the inbred lines (Mahama et al., 2014). Increased N fertilization in sorghum improved panicle number, grain number per panicle, kernel size, grain weight, and harvest index (Buah & Mwinkaara, 2009; Buah et al., 2012; Mousavi et al., 2012; Worland et al., 2017). However, none of these studies have looked at the effect of a range of combinations of N rates and in-season split applications at critical growth stages on grain yield and intra-panicle variations in kernel number and weight. This is important because N availability influences kernel number, weight, and protein content; hence, N application at these stages could affect yield, protein, or both. Recently, Adotey et al. (2021) reported a significant variation in intra-panicle grain number in sorghum exposed to water-deficit stress. The study concluded that the impact of water-deficit stress was minimal on grains at the top portion of the panicle and was significantly higher on grains in middle and bottom portions of the panicle. This is mainly due to the diminishing assimilate supply in a basipetal grain filling (GF) under water-deficit stress conditions (Adotey et al., 2021). A similar phenomenon has been observed in rice under heat stress conditions, wherein the bottom portion of the grains on the panicle was poorly filled compared to the top and middle (Sathishraj et al., 2016). A similar experimental approach can be used to explore potential opportunities to enhance kernel dynamics in different portions of the panicle by optimizing the rate and timing of N application, which could lead to efficient use of N and enhance yield even under current management practices.

There are many different end uses of sorghum, such as the grain is used for biofuel production and animal feed and is a staple food for low-income countries, whereas the vegetative plant parts can be used for biofuel production, animal feed, or left on the field for nutrient and carbon cycling (Ciampitti & Prasad, 2019). Improving sorghum grain quality, especially protein content, would enhance end use quality and consumer acceptance. Though protein content in sorghum is comparable to that of other cereals like maize and wheat, presence of anti-nutritional factors, such as tannins, polyphenols, and protein cross-linking reduces sorghum protein digestibility in humans and animals (Duodu et al., 2003). A linear increase in sorghum grain protein content with an increase in N supply has been noticed in several studies (Diallo, 2012; Kaufman et al., 2013; Rashid et al., 2008).

Therefore, this study aims to understand the impact of in-season split application of N on NUE, grain yield and protein content, as well as the source–sink dynamics in a common, commercially grown grain sorghum hybrid (Pioneer 86P20) in the High Plains region of the United States. Specific objectives addressed in this study are to (1) identify the optimum rate and timing of N fertilization that would maximize sorghum grain yield and quality under different soil types; (2) assess the effect of rate and timing of N fertilization on intra-panicle grain number, grain weight, protein and starch accumulation dynamics; and (3) explore the temporal relationship between sensor-based high-throughput phenotyping and NUE in sorghum.

2 | MATERIALS AND METHODS

2.1 | Experimental sites, nitrogen application, and treatments

Field experiments were conducted during the growing seasons of 2020 and 2021 at the Ashland Bottoms Research Farm, Kansas State

University, Manhattan, KS, USA (9638′7″ W, 397′33″ N). Experiments included three environments with two contrasting soil types, wherein environment 1 (2020) and environment 2 (2021) consisted of Belvue silt loam and environment 3 (2021) had Wymore silty clay loam (Figure S1). Environments 1 and 3 had a previous crop of soybeans, whereas environment 2 had winter wheat as the preceding crop. A popular grain sorghum hybrid for the region, Pioneer 86P20, was grown across all environments. The three environments were located within 2.0 km of the KSU Mesonet, Ashland Bottoms (http://mesonet.k-state.edu/) weather station, which recorded all climatic data. The mean daily air temperature and cumulative precipitation of the two growing seasons can be visualized in Figure S2. Recommended cultivation, herbicide, and pesticide management practices for the region were followed. The experiments were laid out in a randomized complete block design with 9 N application treatments replicated four times per environment with a plot size of 46.33 m^2 (four rows, 76.2 cm spacing between rows, with a row length of 15.2 m). A seeding rate of approx. 144,800 seeds ha^{-1} was sown in each environment, where environment 1 was planted on May 20, 2020, and both environments 2 and 3 were planted on June 07, 2021. Each plot was separated by a 3.0 m border to prevent seepage of N from one plot to another (Figure S3). Soil samples were collected within 30 days prior to planting at each environment; two soil cores per plot were extracted at depths of 0–15 and 15–30 cm, individually. Cores collected at corresponding soil depths within each plot were combined (two samples per plot) and submitted to the Kansas State University soil-testing laboratory for soil physical parameters and nutrient analysis. Once at the lab, the soil samples were transferred to sterile, labeled containers and dried in a forced air oven. Once dry, they were ground with a pulverizing-type grinder and passed through a 2-mm sieve. A 1:1 soil:water Sikora buffer procedure was used to obtain the soil pH, whereas the modified Walkley–Black method was used to estimate soil organic matter. To obtain soil mineral N amount (nitrate and ammonia), the KCl extraction method was used, and the Mehlich-3 extraction procedure was used for phosphorus. Finally, to estimate potassium, the ammonium acetate extraction method was used.

Treatments included a 0 N control where no N was applied (N0), seven treatments that varied in N application amounts (N31–N170) and timing, and one sensor-based treatment (N170) (Table 1). Liquid urea-ammonium nitrate (28-0-0 N-P-K) was used as the N source. The sensor-based N application treatment utilized a CropSpec (Topcon Positioning Systems, Inc.) sensor, which performed a target variable rate based on the reflectance of plant material within the plot. Each treatment, except N0, had 31 kg N ha^{-1} applied at the time of planting. Treatments N65, N64, N92, N125, N130, N157, and N170 each included varying N application amounts at key physiological growth stages (GS3—panicle initiation and GS5—booting). The application of N was completed using streamer nozzles with 76.2 cm spacing attached to 38.1 cm drops. The nozzles were spaced so that they would travel between rows of plants, and drops were located such that the nozzles were below the canopy to minimize the damage of aboveground plant tissues from the fertilizer. The applicator setup is shown in Figure S4.

TABLE 1 Applied nitrogen in each treatment at different timings, averaged across all three environments.

	Nitrogen application timing and amount (kg N ha^{-1})			
Treatment	**Planting**	**GS3**	**GS5**	**Total**
N0	0.0	0.0	0.0	0.0
N31	31.4	0.0	0.0	31.4
N65	32.0	33.0	0.0	65.0
N64	31.5	0.0	32.0	63.5
N92	31.0	30.2	31.4	92.6
N125	32.1	61.1	32.2	125.4
N130	32.9	34.1	63.1	130.2
N157	32.8	59.9	63.9	156.6
N170	33.7	94.5	41.8	170.1

Note: Treatment column shows the total amount of nitrogen applied per corresponding treatment (kg N ha^{-1}). Planting—application at planting; GS3—application at panicle initiation; and GS5—application at booting.

2.2 | Parameters recorded

2.2.1 | Plant population and biomass

After plant emergence, plant stand counts were recorded per plot to account for differences in plant density. Whole plant biomass samples (0.5 m of either interior two rows) were harvested manually at the ground level in each plot at two different growth stages: mid-GF (MGF) (GS6) and physiological maturity (GS9). Each of these samplings were done on a per plot basis per replication across all three environments, totaling 36 samples per environment. The samples were immediately separated into stems, leaves, and panicles prior to air drying at 60°C to constant weight. The separated samples were then weighed individually to obtain plant part biomass and summed for whole shoot biomass.

2.2.2 | Aerial- and ground-based sensor data and chlorophyll index

Aerial reflectance data throughout the growing season was measured using a DJI Mavic 210 RTK quadcopter (SZ DJI Technology Co.), which was equipped with a SlantRange 4p+ (SlantRange Inc.) multispectral sensor. Data capture time points were scheduled twice (between 10:00 and 14:00) per week throughout the growing season, except under challenging climate conditions such as high wind speed or partly cloudy skies. The 4p+ captured spectral bands of red, green, blue, red (RGB) edge, and near-infrared, at 670, 470, 520, 720, and 850 nm, respectively. The quadcopter was flown at an aboveground level of 40 m and at a horizontal speed of 1.7 m s^{-1}. This provided a 75% forward, rear, and side overlap of images with a ground sampling distance of 0.7 cm per pixel. The raw images were initially processed in SlantView

2 (SlantRange image analysis software) for preparation for processing in Pix4D Mapper (Pix4D S.A.). Digital elevation models were then created in Pix4D and orthomosaics were outputted for further processing in ArcGIS Pro (ESRI). The RGB images were used to classify the orthomosaics into four categories, where applicable (soil, shadow, plant, and panicle), using a support vector machine algorithm within ArcGIS Pro. For the classification of each category, ≥20 training samples were collected from each data capture time point and created separately for the greatest accuracy. The red, red edge, and near-infrared bands were then utilized to calculate normalized difference vegetation index (NDVI) and normalized difference red edge index (NDRE) rasters (formulae listed below). Polygons for each individual plot harvest area were created, and orthomosaics and classified rasters were then clipped to the harvest area polygons. Areas that were classified as plants were then extracted to obtain the NDVI and NDRE from only the plant leaf material. To compare reflectance indices to phenology, growing degree units (GDU) were estimated using the hourly thermal time (HTT) method as described by Ritchie and Nesmith (1991):

$$NDVI = \frac{NIR - R}{NIR + R}, \tag{1}$$

$$NDRE = \frac{NIR - RE}{NIR + RE}, \tag{2}$$

$$HTT = \begin{cases} 0 \quad T_h < T_b \\ T_h - T \quad T_b \leq T_h \leq T_{opt} \\ \frac{T_{opt}-T_b}{T_u-T_{opt}}\,(T_u - T_h) \quad T_{opt} < T_h \leq T_u \\ 0 \quad T_u < T_h \end{cases}, \tag{3}$$

$$GDU = \left(\sum_{1}^{24} HTT_i\right)/24, \tag{4}$$

where *NDVI* is normalized difference vegetation index, *NIR* is near infrared band, *R* is red band, *NDRE* is normalized difference red edge, *RE* is red edge band, *HTT* is hourly thermal time, T_h is hourly temperature, T_b is base temperature, T_{opt} is optimum temperature, and T_u is maximum temperature.

T_b was set at 10.0 and 5.7°C, T_{opt} was set at 30.0 and 23.5°C for pre- and post-flowering GDU calculations, respectively, whereas T_u was set at 42.0°C for both stages following Ostmeyer et al. (2020).

Chlorophyll index (using soil plant analyzer development, SPAD; Model 502; Spectrum Technologies) was recorded on three areas of the flag leaf (basal, middle, and tip) of three random plants within the harvest area of each plot at early GF (EGF), MGF, and late GF (LGF) periods. The nine recorded chlorophyll index values of each plot at a growth stage were averaged for a plot mean.

2.2.3 | Whole plant leaf nitrogen, grain yield, and quality parameters

The dried leaves from the 0.5 m sample at maturity were ground to pass through a 2-mm sieve and submitted to the Kansas State University soil-testing laboratory for N content analysis through the dry combustion method. Leaf N content (LNC) was used to extrapolate the estimated residual leaf N (RLN) on an area basis by multiplying the percent LNC by the leaf biomass per unit area. Grain yield was recorded using a two-row plot harvester where only the middle two rows of the four-row plot were harvested (23.16 m^2) to avoid any confounding effects from the border rows (Figure S3). The area used for the 0.5 m row biomass samplings was excluded when calculating the plot yield on an area basis.

Panicles from the 0.5 m mature plants collected in each environment were threshed and cleaned to obtain grain weight, thousand-grain weight, and harvest index. All the bulk threshed grains were run through an NIR using standardized curves for both grain protein (calibration [$R^2 = 0.94$] and validation [$R^2 = 0.90$]) and starch (calibration [$R^2 = 0.87$] and validation [$R^2 = 0.76$]) (Peiris et al., 2019, 2021).

2.2.4 | Intra-panicle grain number and weight

Four random, representative panicles from the mature 0.5 m samples from environments 2 and 3 were set aside for panicle partitioning to observe differences in within panicle grain characteristics. Each individual panicle was measured for total panicle length (from the tip to the bottom-most rachis and then divided into three portions based on the total length (top, middle, and bottom). Further, the panicle was sectioned along the main stem of the panicle at the one-third length, with the rachis located at the node below and above the one-third length were kept with the corresponding stem portions (Figure S5). This sectioning method has been used to determine the intra-panicle GF dynamics under water-deficit stress in sorghum (Adotey et al., 2021). Each portion was individually hand threshed and cleaned. The portioned panicle grains were counted using an electronic seed counter (Key-Mat Equipment) and weighed.

2.2.5 | Plant nitrogen use efficiencies

Total grain protein content values were used to estimate the grain N content using the 6.25 Jones factor. Grain N content, applied N amounts, and grain yields were used to estimate percent N recovery (percent fertilizer recovery [PFR]), agronomic efficiency, partial nutrient balance, and partial factor productivity. Formulae are as follows:

$$Grain\ nitrogen\ recovery\ (\%) = \frac{GN_a - GN_c}{N}, \tag{5}$$

$$Agronomic\ efficiency = \frac{GY_a - GY_c}{N}, \tag{6}$$

$$Partial\ nutrient\ balance = \frac{GNa}{N}, \tag{7}$$

$$Partial\ factor\ productivity = \frac{GYa}{N}, \tag{8}$$

where GN_a is the grain N content of N application treatment, GN_c is the grain N content of zero N application (control treatment), N is the total N applied, GY_a is the grain yield of N application treatment, and GY_c is the grain yield of zero N application (control treatment).

These four N use efficiency formulae were modified from Hawkesford (2012).

2.3 | Statistical analysis

All the data were tested for their normal distribution through the Kolmogorov–Smirnov test using the PROC UNIVARIATE option in SAS software (Version 9.4, SAS Institute). Two-way analysis of variance (ANOVA) was performed for all traits using N treatment and environment as the two factors (Table S1). Three-way ANOVA was performed for SPAD, and biomass N treatment, environment, and time of measurement or sampling date as three factors (Table S1). Three-way ANOVA was also performed for portioned panicle data using N treatment, environment, and panicle portion as three factors (Table S2). Repeated measures ANOVA was performed for NDVI and NDRE recorded over different time points, using the PROC MIXED procedure in SAS software, using the first-order autoregressive covariance matrix structure (Table S1). Significant mean separation between treatments or environments was determined using Tukey's honest significant test (HSD). Pearson correlation analysis was performed using the PROC CORR procedure in SAS (Version 9.4, SAS Institute). The two- and three-way ANOVA and HSD tests were performed using the PROC MIXED procedure in SAS software.

3 | RESULTS

3.1 | Soil characteristic, weather parameters, and nutrient analysis

Mean soil mineral N averaged across all plots per environment before planting is shown in Figure S1, where 94.6, 26.2, and 19.3 kg N ha^{-1} were found in environments 1–3, respectively. Data on soil pH, organic matter, nitrate, ammonium, available phosphorus, and exchangeable potassium of each environment and treatment are presented in Table S3. Environment 1 had higher NO_3-N content at both 0–15 and 15–30 cm soil depths than other two environments. Environment 2 had lower phosphorus than other environments, whereas environment 3 had lower organic matter and high phosphorus than other environments (Table S3). Average and range of daily mean temperature, relative humidity, precipitation, and solar radiation are given in Table S4. All the measured weather parameters remained similar between the 2 years except that the average daily precipitation or rainfall was 1 mm less in year 2021 than year 2020 (Table S4).

3.2 | Plant biomass, yield, and yield parameters

A summary of ANOVA of all traits recorded is presented in Table S1. There was no significant difference between treatments for plant population (plants ha^{-1}; Table S1). All the biomass parameters measured (leaf, stem, panicle, and aboveground biomass) varied significantly ($p < 0.001$) between environments and time of sampling but not for treatment. Significant N treatment and environment interaction effect was noticed for leaf biomass. There was no significant three-way interaction effect (N treatment × time of sampling × environment) for biomass traits (Table S1). Grain yield varied significantly ($p < 0.001$) between environments with an average grain yield of 7.5 t ha^{-1} in environment 1, 4.9 t ha^{-1} in environment 2, and 7.1 t ha^{-1} in environment 3 (Table S5). Grain yield averaged across environments generally recorded an increasing trend with increase in total N applied but did not vary significantly between treatments (Table S5). However, grain yield was significantly affected by N treatment in environments 2 ($p < 0.001$) and 3 ($p = 0.032$) but not in environment 1. In environment 2, N170 showed a significant ($p < 0.05$) increase of 30.5% in grain yield compared to N0. In environment 3, grain yield under N170 was 19.4% higher than N0 (Table S5). Thousand-grain weight, aboveground biomass, and harvest index did not vary significantly between N treatments or their interactions with environment (Tables S1 and 5).

3.3 | Chlorophyll index, NDVI, and NDRE

SPAD, NDVI, and NDRE were significantly ($p < 0.05$) affected by N treatment, environment, and the time of measurement (Table S1). A significant three-way interaction (N treatment × time of measurement × environment) effect was noticed only for NDRE. Significant difference between treatments and environments was recorded for flag leaf chlorophyll index (SPAD; $p < 0.001$), NDVI ($p < 0.05$), and NDRE ($p < 0.001$) at EGF, MGF, and LGF growth stages, except for NDVI and mid-grain fill (Table S1). The five treatments in which the highest amount of total N was applied (N92, N125, N130, N157, and N170) showed the highest SPAD values across the entire GF period (Figure 1). Treatment N0 recorded significantly ($p < 0.05$) lower chlorophyll index than all other higher N treatments from N125 to N170, at all the three growth stages (Figure 1). NDVI values at high N treatments, that is, N157 and N170, were significantly ($p < 0.05$) higher than N0 at EGF and LGF stages (Figure 2A,C). Higher N treatments like N157 and N170 recorded significantly ($p < 0.05$) higher NDRE values than N0 and N31 throughout the GF period (Figure 2D–F).

When temporal NDVI and NDRE values across the growing season are plotted (Figure 3), a separation of the two differing split application treatments (N65 and N64) was noticed with N65 recording a higher NDVI and NDRE throughout the GF period than N64 in environment 1. Treatment N65 with N applied at basal + GS3 recorded an increase in NDVI and NDRE values soon after N application at GS3 than that of the later applied treatment (N64) (Figure 3). Higher N treatment N157

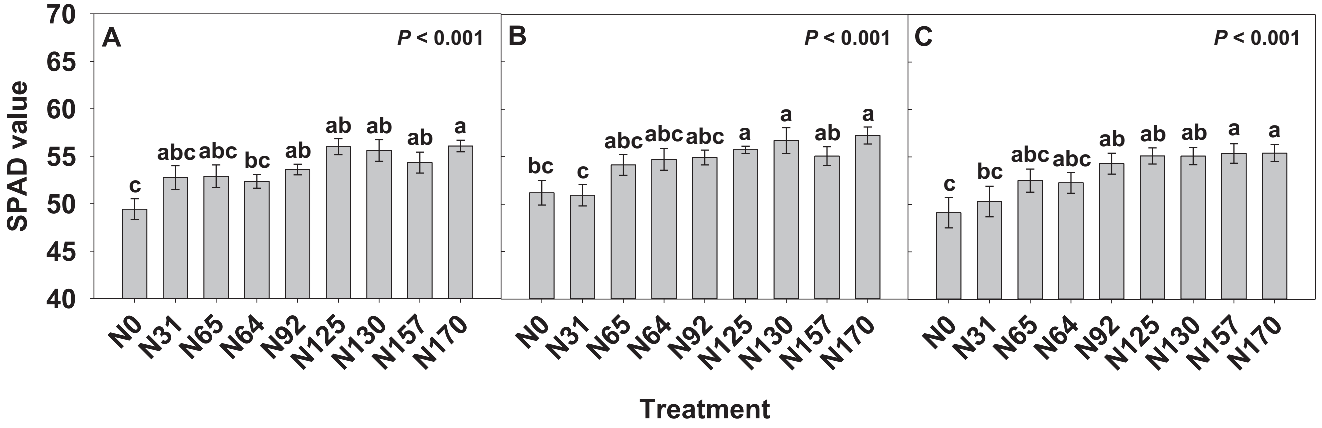

FIGURE 1 Chlorophyll index (SPAD) values during the early- (A), mid- (B), and late- (C) grain-filling stages. Bars represent mean values per treatment across environments ± standard error ($n = 12$). Different letters represent significant differences between treatments per grain-filling stage ($p \leq 0.05$).

recorded higher NDVI and NDRE values than all the other treatments throughout most of the growing period (Figure 3). NDVI recorded a significant positive correlation with grain yield throughout the GF period with the highest correlation coefficient ($r = 0.87$; $p < 0.001$) at MGF (Table 2), whereas NDRE was significantly correlated with grain yield only at MGF and LGF stages with highest correlation at MGF ($r = 0.46$; $p < 0.001$). Chlorophyll index had the highest correlation with yield at LGF period ($r = 0.53$; $p < 0.001$; Table 2).

3.4 | Leaf nitrogen content and whole plot residual leaf nitrogen

Single LNC and whole plot RLN at harvest (N content converted to units per area leaf biomass) showed highly significant ($p < 0.001$) differences among treatments, environments, and their interactions (Table S1). Both LNC and RLN were significantly ($p < 0.05$) high in environment 1 compared to other two environments. LNC and RLN did

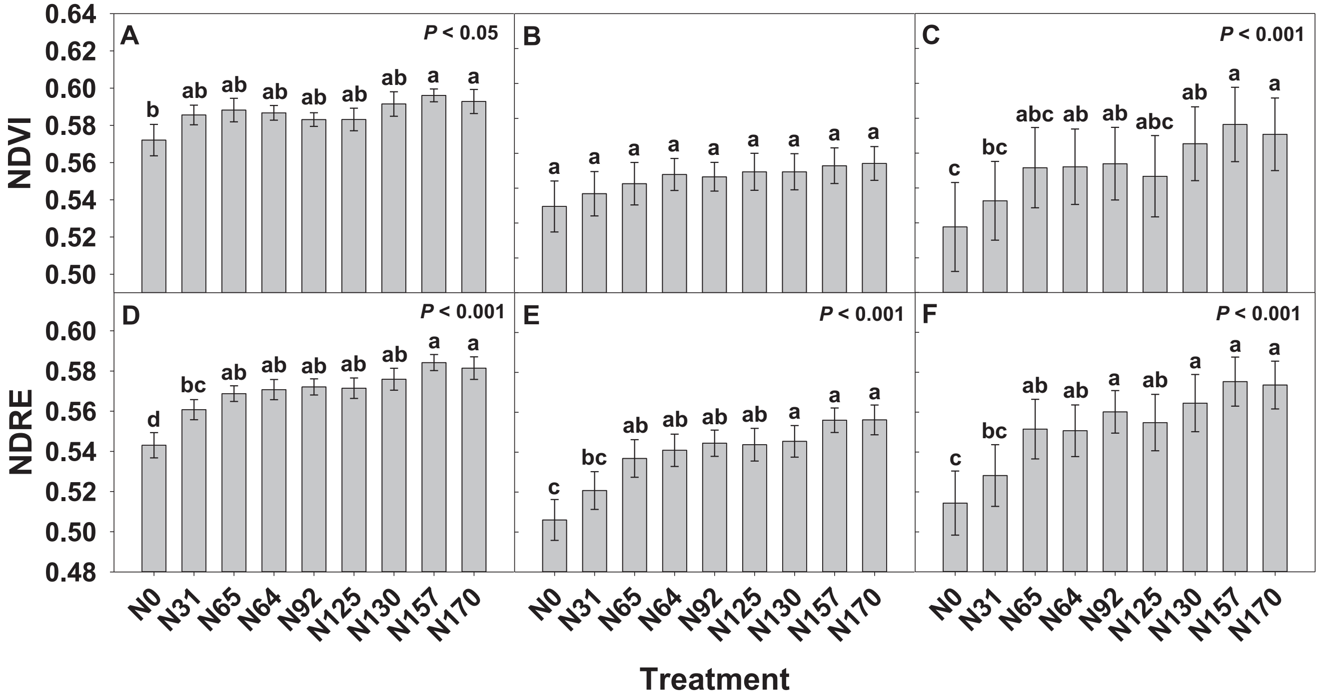

FIGURE 2 Normalized difference vegetative index (NDVI; A–C) and normalized difference red edge (NDRE; D–F) values during the early- (A and D), mid- (B and E), and late- (C and F) grain-filling stages. Bars represent mean values per treatment ± standard error ($n = 12$). Different letters represent significant difference between treatments ($p \leq 0.05$).

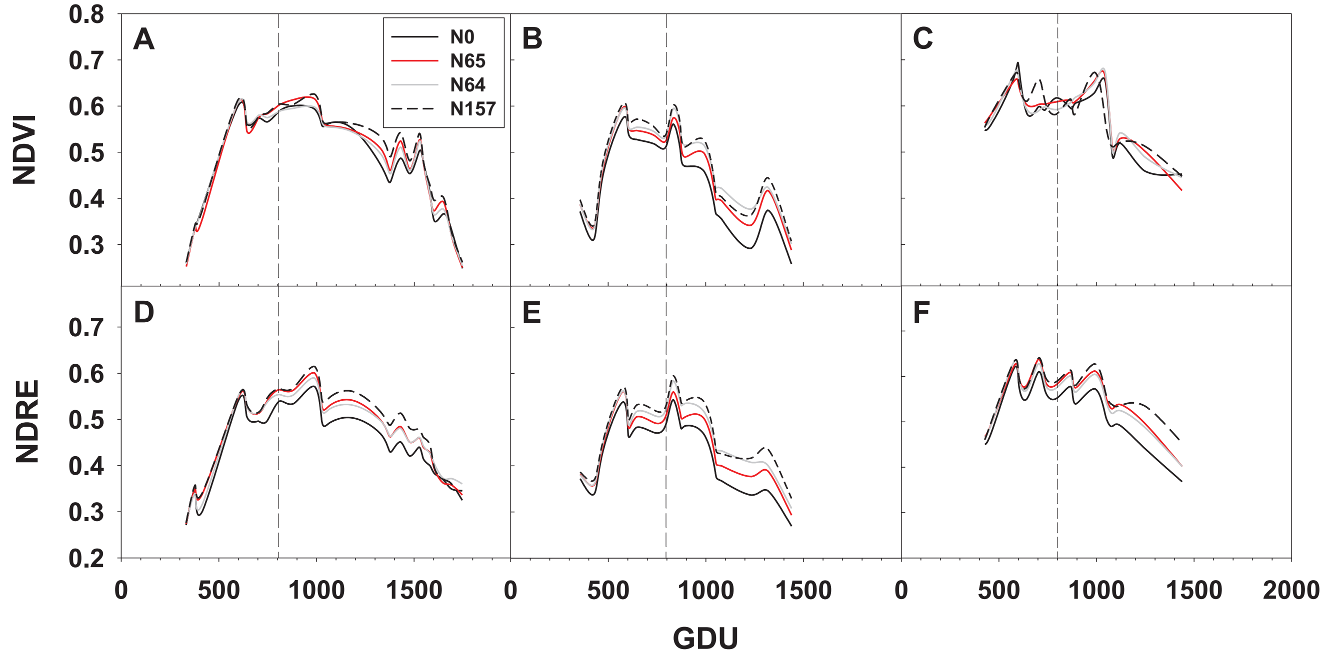

FIGURE 3 Temporal normalized difference vegetative index (NDVI; A–C) and normalized difference red edge (NDRE: D-F) of four contrasting treatments (N0, N65, N64, and N157) across the growing season (GDU: growing degree units) in environments 1 (A and D: 2020), 2 (B and E; 2021), and 3 (C and F; 2021). The vertical dashed line represents 50% anthesis in all environments (800 GDU).

not vary significantly across N treatments in environment 1, which is mainly due to the high available soil N (94.6 kg N ha^{-1}) in this environment compared to other environments (Figure 4A,B; Figure S1). However, in environments 2 and 3, a significant ($p < 0.05$) variation was noticed for LNC and RLN across N treatments (Figure 4C–F). With the increase of total N applied, LNC also increased linearly, ranging from 1.46% to 1.95% in environments 2 and 3 (Figure 4C,E). In environments 1 and 3, treatment N64 with split application at GS5 recorded a decrease in LNC compared to N65 (2.0%–2.1% and 1.4%–1.6%, respectively) (Figure 4A,E). Higher N treatments N157 and N170 had significantly ($p < 0.05$) higher LNC than lower N treatments, that is, N0 up to N65 in environments 2 and 3 (Figure 4C,E). In environment 3, N92 showed significantly lower LNC and RLN than N170 (Figure 4E,F). LNC correlated significantly with N application amounts at panicle initiation ($r = 0.45$; $p < 0.001$), booting ($r = 0.39$; $p < 0.001$), and planting ($r = 0.50$; $p < 0.001$; data not shown). LNC was also significantly correlated to grain yield ($r = 0.62$; $p < 0.001$) and protein content ($r = 0.64$; $p < 0.001$; data not shown).

TABLE 2 Correlation between chlorophyll index (SPAD), normalized difference vegetative indices (NDVI) and normalized difference red edge (NDRE) versus grain yield (t ha^{-1}) and protein content (%; dwb: dry weight basis) averaged across three environments at three stages during the grain-filling period.

Timing	Indices	Yield	Protein
Early grain filling	SPAD	0.38***	0.45***
	NDVI	0.34***	0.29**
	NDRE	0.08	0.44***
Mid grain filling	SPAD	0.48***	0.47***
	NDVI	0.87***	0.35***
	NDRE	0.46***	0.47***
Late grain filling	SPAD	0.53***	0.53***
	NDVI	0.37***	0.21*
	NDRE	0.34***	0.34***

*$p \leq 0.05$.
**$p \leq 0.01$.
***$p \leq 0.001$.

3.5 | Intra-panicle grain partitioning

Grain weight, number, and individual kernel weight were significantly affected by environment ($p < 0.001$), portion ($p < 0.001$), and environment × portion ($p < 0.05$) interaction but not by treatment and its interaction effects (Table S2). A significantly ($p < 0.05$) higher number of grains were found in the bottom portion of the panicle compared to the top in all panicles irrespective of the treatment (Figure 5A). A similar trend was seen in grain weight (Figure 5B), whereas an inverse trend was observed for the individual kernel weight (Figure 5C). Kernel weight was significantly ($p < 0.05$) lower in grains at the bottom portion of the panicle compared to the top portion in almost all treatments (Figure 5C).

Mean values of the intra-panicle grain numbers between two environments in 2021 were significantly ($p < 0.05$) lower in environment 2 (lower yielding environment) in all three portions of the panicle

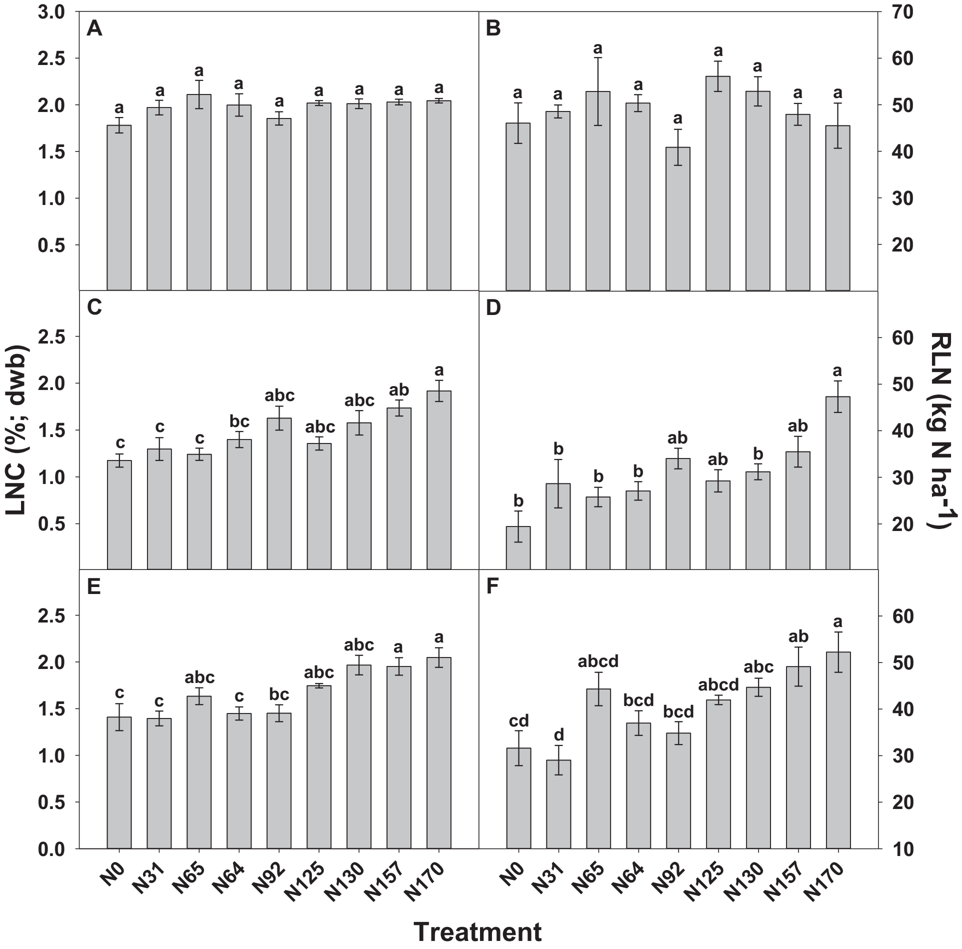

FIGURE 4 Leaf nitrogen content (LNC in %; dwb: dry weight basis; A, C, and E) and residual plot leaf nitrogen (RLN in kg N ha^{-1}; B, D, and F) at plant maturity in environments 1 (A and B), 2 (C and D), and 3 (E and F). Bars represent mean values per treatment ± standard error ($n = 4$). Different letters represent significant difference between treatments ($p \leq 0.05$).

compared to environment 3 (Figure 6A), whereas the intra-panicle individual kernel weight of the bottom portion of the panicle was significantly ($p < 0.05$) lower in environment 2 compared to environment 3 (Figure 6B).

3.6 | Total protein and starch content

Total protein and starch content in the grain was significantly affected by treatment ($p < 0.05$) and environment ($p < 0.001$), but not by their interaction (Table S1). The grain protein content increased with increasing N application in all the three environments (Figure 7A,C,E). In contrast, starch content decreased significantly ($p < 0.05$) with increased N application in environments 2 and 3 (Figure 7D,F). Enhanced protein accumulation under high N application reduced the space for starch accumulation in the grains (Figure 7). There was no significant difference in both protein and starch content between N treatments with similar N rate and different application timings (N64 vs. N65 and N125 vs. N130) (Figure 7). NDVI and NDRE recorded a significant ($p < 0.05$) positive correlation with protein content throughout the GF period (Table 2). In all three periods of GF, NDRE exhibited highest correlation with protein content compared to NDVI, with highest correlation observed at MGF period. Thus, when predicting grain protein with high-throughput phenotyping, NDRE appears to be the better vegetative index compared to NDVI.

3.7 | Nitrogen use efficiency

Agronomic efficiency, partial nutrient balance (PNB), and partial factor productivity (PFP) showed a significant ($p < 0.05$) treatment, environment, and treatment × environment interaction effect (Table S1). Soil mineral N prior to application had a major impact on the fertilizer recovery percent and the agronomic efficiency. Environment 1 had a significantly ($p < 0.05$) higher amount of soil N than that of the other two environments, resulting in significantly ($p < 0.05$) lower PFR and AE (Figure 8A,B). PFR was significantly ($p < 0.05$) higher in environment 3 compared to environments 1 and 2 (Figure 8A). PNB and PFP followed the trend of decreased efficiency with higher amounts of N applied (Figure 9).

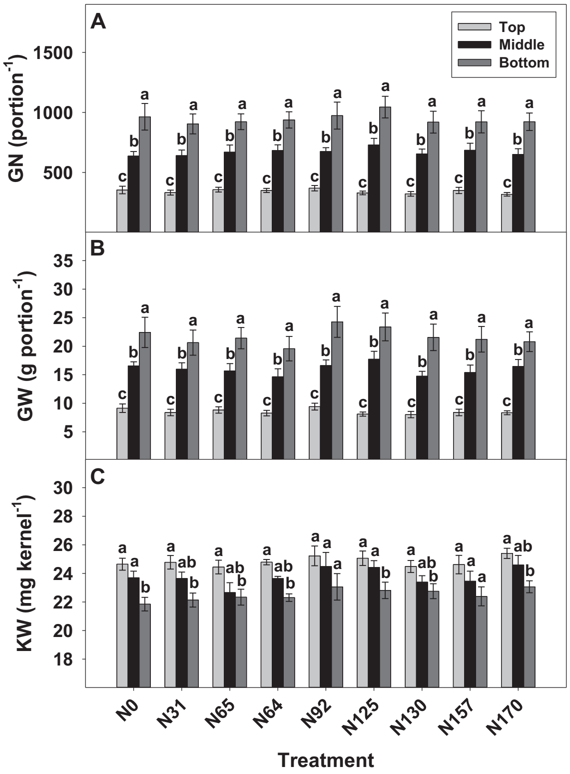

FIGURE 5 Intra-panicle grain number (GN; A), grain weight (GW; B), and kernel weight (KW; C) of top, middle, and bottom portions of the panicle across nine nitrogen application treatments combined from two environments in 2021. Bars represent mean values per portion across treatments ± standard error ($n = 8$). Different letters represent significant difference between portions of panicle within the same treatments ($p \leq 0.05$).

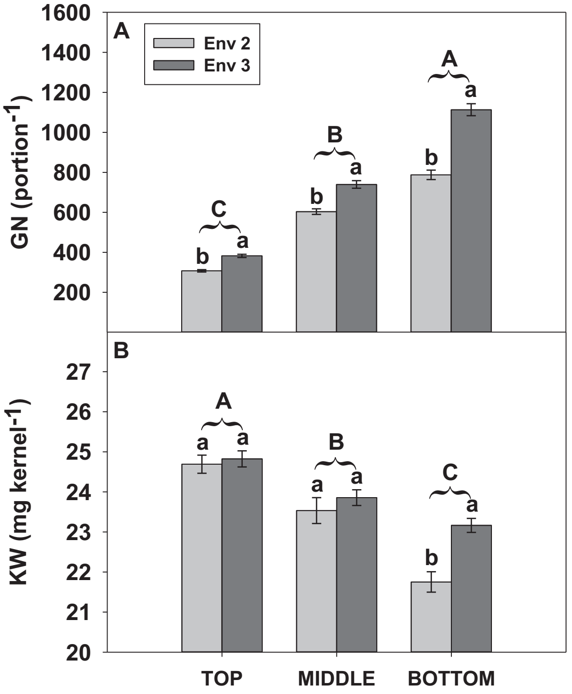

FIGURE 6 Mean intra-panicle grain number (GN by portion of panicle; A) and kernel weight (KW by portion of panicle; B) of top, middle, and bottom portions of the panicle across nine nitrogen application treatments combined but separated by two environments in 2021. Bars represent mean values per portion across environments ± standard error ($n = 16$). Different uppercase letters represent significant difference between portions of panicle, whereas different lowercase letters represent significant difference between environments within respective portion ($p \leq 0.05$).

4 | DISCUSSIONS

4.1 | Optimum amount and timing of N fertilization improves grain yield and quality in sorghum

Optimal fertilizer rates for maximum yield have been previously documented (Mahama et al., 2014) but are typically considered on the traditional practice of complete N fertilization at planting, or even earlier. Theoretically, the split application of N at key growth stages at which plants take up N at high rates will increase yield and quality while reducing N losses (Yang et al., 2021). Grain yield varied significantly ($p < 0.01$) between N treatments in environments 2 and 3 with low soil mineral N (Table S5), whereas a high soil mineral N of 95 kg N ha^{-1} provided enough N for plant uptake and masked the effect of N treatments on grain yield. Although shoot biomass across all three environments in this study was not significantly impacted (Table S5), grain protein content increased significantly ($p < 0.05$) in high N fertilization treatments (Figure 7A,C,E). This indicates that plants continue to uptake additional N and translocate it to the grain. Interestingly, even in environment 1, where soil mineral N was high (>90 kg N ha^{-1}), a grain protein response to N treatment was found (Figure 7A). Though nonsignificant, the earlier applied N in N65 (panicle initiation) resulted in an increase in protein content compared to N64 (booting), despite the total N applied being same in both these treatments (Figure 7A,C,E). However, the application of N at both panicle initiation and booting (N92) increased protein content compared to N65 and N64 treatments. These results suggest that N fertilization at panicle initiation is more critical than at booting, but with the application at both timings, protein can be further enhanced. Further increase in the amount of N applied from N92 to N170 did not have any beneficial effect on protein content indicating that split application of 31 kg N ha^{-1} each at the time of planting, panicle initiation, and booting is more efficient and beneficial rather than increasing the N amount. The above findings indicate that the in-season split application of N enhances grain yield under low soil mineral N and improves protein content irrespective of soil N status. Split application of 31 kg N ha^{-1} each at planting, panicle initiation, and booting is an optimum N treatment for increasing protein content in sorghum.

4.2 | Opportunity to enhance grain yield by targeting lower portion of sorghum panicle

Though environment 3 recorded a significant ($p < 0.05$) increase in grain number at all the three portions of the panicle compared to

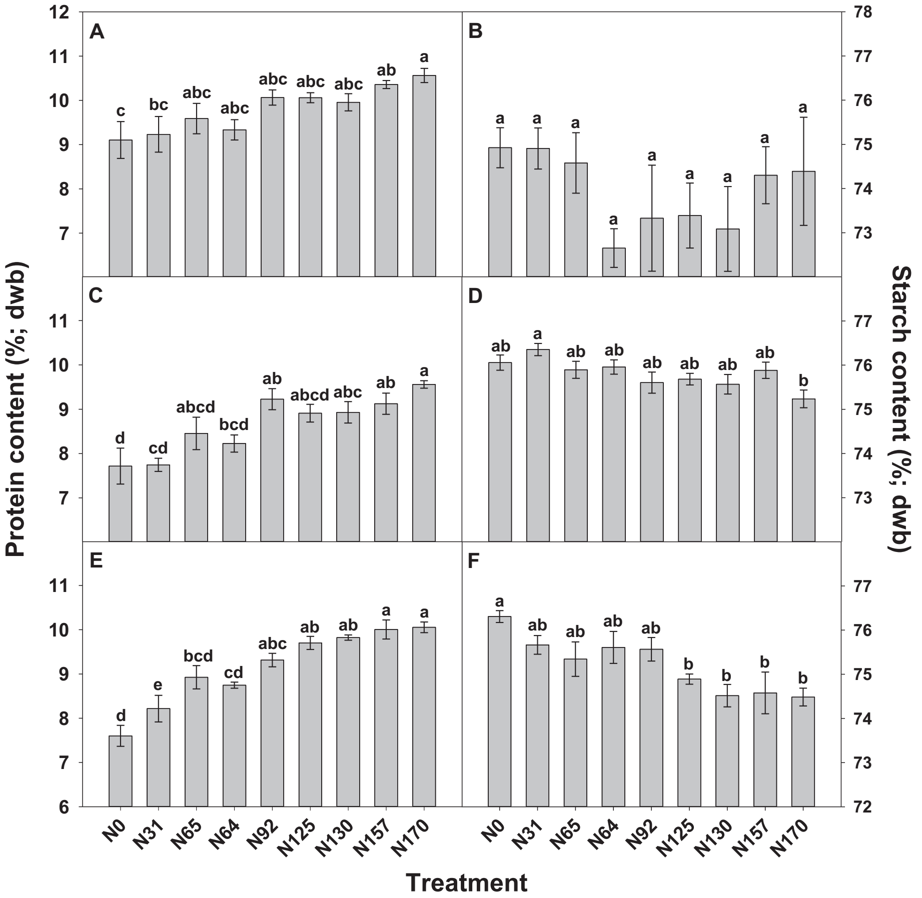

FIGURE 7 Protein content (A, C, E) and starch content (B, D, F) in environments 1 (A and B), 2 (C and D), and 3 (E and F); dwb: dry weight basis. Bars represent mean values per treatment ± standard error ($n = 4$). Different letters represent significant difference between treatments ($p \leq 0.05$).

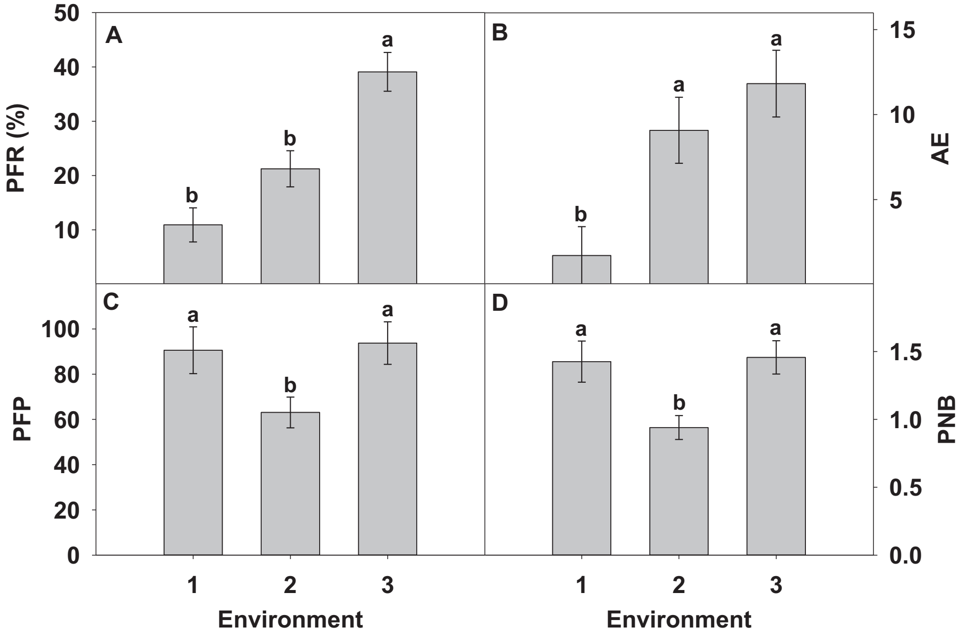

FIGURE 8 Percent fertilizer recovery (A), agronomic efficiency (B), partial factor productivity (C), and partial nutrient balance (D) across three environments averaged across all nitrogen application treatments. Bars represent mean values per environment ± standard error ($n = 32$). Different letters represent significant difference between environments ($p < 0.05$).

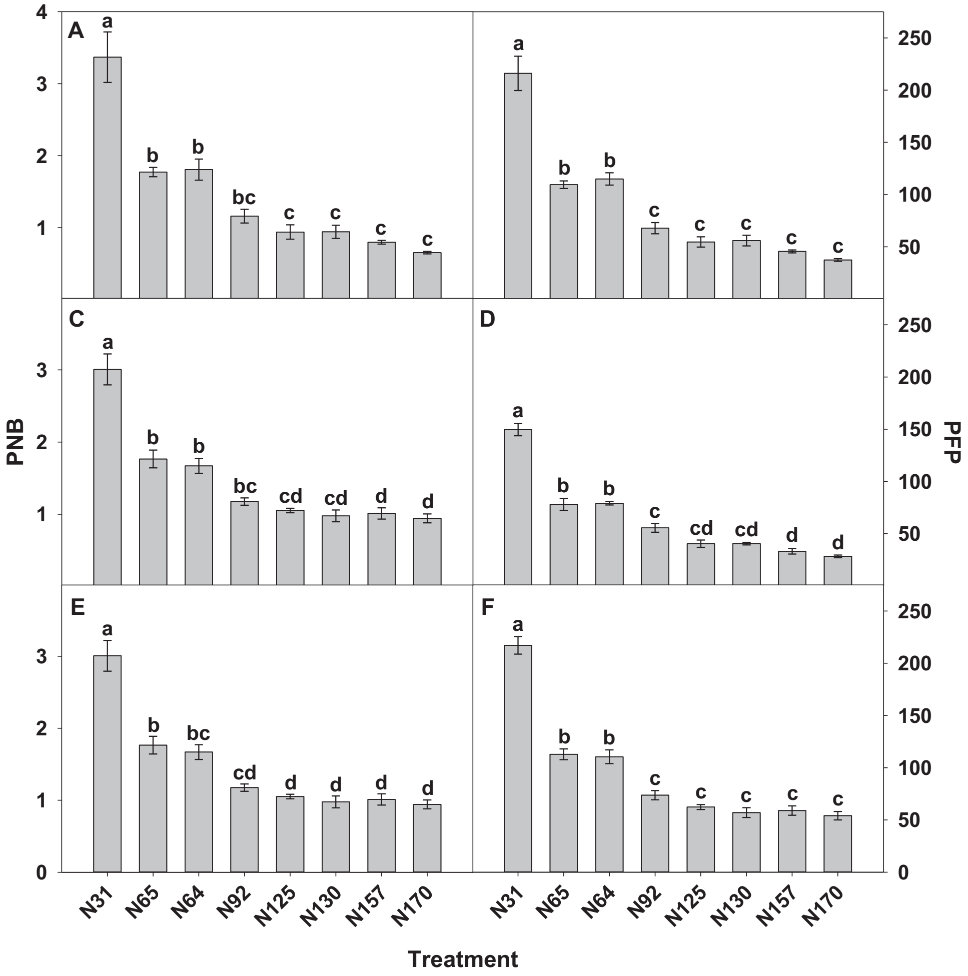

FIGURE 9 Partial nutrient balance (PNB; A, C, and E) and partial factor productivity (PFP; B, D, and F) of nitrogen application treatments in environments 1 (A and B), 2 (C and D), and 3 (E and F). Bars represent mean values per treatment ± standard error ($n = 4$). Different letters represent significant difference between treatments ($p < 0.05$).

environment 2 (Figure 6A), kernel weight was significantly ($p < 0.05$) higher in environment 3 only at the bottom portion of the panicle (Figure 6B). This indicates that, irrespective of grain number, grains at the top and middle portions of the panicle were able to efficiently fill all the grains, but not the grains at the bottom portion of the panicle. In addition, a significantly ($p < 0.05$) lower kernel weight of individual grains at the bottom portion of the panicle compared to top was noticed in all the N treatments, except N92 and N157 (Figure 5C). This shows that grain yield can be potentially increased with breeding for higher grain weight kernels at the bottom portion of the panicle. The phenomenon of superior (florets at the top third of the panicle) and inferior (florets at the bottom third) spikelets within the panicle is well established in rice (Hongthong et al., 2012; Mohapatra et al., 2011; Sathishraj et al., 2016) and recently in sorghum under drought (Adotey et al., 2021). This is attributed to the florets at the top third having access to a larger assimilate pool compared to the florets at the bottom portion that flower late resulting in lesser access to assimilates (You et al., 2016), as justified in crops such as rice, which has strong pre-harvest senescence characteristics (Biswas & Choudhuri, 1980). However, the large amounts of residual N in sorghum, even at harvest (Figure 4), indicates that the lower grain weight in grains at the bottom portion of the panicle may not be due to limited assimilates. This could either be due to a reduced rate of assimilate supply toward the end of the GF phase associated with extended stay-green phenomenon even at harvest, or dynamic interaction between grain number and weight influenced by panicle architecture. Though this requires additional investigation, results presented here indicate that breeding for increased grain weight in the bottom portion of the panicle could benefit sorghum productivity under relatively favorable conditions.

4.3 | Sensor-based vegetative indices have the potential to be good predictors of grain yield and protein content

High-throughput phenotyping is increasing in popularity due to its ease of use and abundance of data collection at high temporal frequency during the crop-growing season (Zhang & Kovacs, 2012). Utilizing either unmanned aerial vehicles (UAVs) or equipment-mounted sensors like the Topcon CropSpec allows for a nondestructive, on-the-go data capture and analysis for predicting grain yields and grain quality (Zhang & Kovacs, 2012). An equipment-mounted sensor has the capability of adjusting fertilizer application rates as the applicator travels through the field, sensing the crop health or greenness. This allows more fertilizer to be applied to areas needing it, and less applied to areas with lower fertilizer requirement, thus reducing producer inputs costs and lowering fertilizer loss (Yang et al., 2001). These new technologies are used increasingly in breeding programs to make selections and identify certain traits with higher precision than manual measurement (Potgieter et al., 2017). In sorghum, the moderate-to-strong correlations noticed with vegetative indices with late-season biomass, leaf nitrogen, and chlorophyll contents indicated that the vegetative indices measured through UAVs can aid in large-scale phenotyping (Li et al., 2018). In maize, both the vegetative indices NDVI and NDRE were related to N rate across multiple environments (Burns et al., 2022; Shaver et al., 2011). These two UAV-based traits analyzed in this study resulted as powerful predictors of grain yield and protein content. A commonly used vegetative index, NDVI, showed significant ($p < 0.001$) correlation to grain yield, whereas NDRE correlated the greatest with protein content throughout the GF period (Table 2). In sorghum, NDVI was found to be significantly correlated with plant number per plot, canopy cover, and leaf area index during both pre- and post-anthesis periods across locations (Potgieter, et al., 2017; Richardson et al., 1992; Shafian et al., 2018). In addition, NDRE was useful in assessing leaf senescence pattern and chlorophyll content (Li et al., 2018; Potgieter, et al., 2017). In the current study, these vegetative indices prove to be useful tools for early prediction of yield and protein and have the opportunity to be utilized in sorghum-breeding program for the selection of lines for advancement to enhance yield and protein.

4.4 | Influence of residual soil N and differential amount and timing of N fertilization on NUE and sustainability of sorghum

It was visually obvious that the hybrid included in this study (Pioneer 86P20) had a strong stay-green trait. The leaves were healthy and green at the time of harvest in all three environments. The strong stay-green trait in this hybrid could explain the little to no change in yield. Even with little soil residual and no applied N (N0), the hybrid was able to yield at a level with higher applied N. Although the yield was minimally changed from different levels of N application, a significant ($p < 0.05$) difference was found in the grain protein and the whole plant RLN. A 25% increase in LNC was found when comparing the N0 control to the highest applied N treatment (N170), indicating that plants took up available N and stored it in the leaves (Figure 4A). By extrapolating the LNC to a mass per area unit (kg N ha^{-1}), it was apparent that this hybrid is leaving a large amount of stored N in the leaves. The N0 control was still able to take up and store almost equal amount of N within the leaves to that of N170 in environment 1 with high available soil N (Figure 4B), whereas in the other 2 environments, 25–30 kg N ha^{-1} was locked within leaves under N0 (Figure 4D,F) as compared to 45–55 kg N ha^{-1} under N170. These results highlight the sustainable characteristics of grain sorghum. When in a crop rotation, harvesting a quality yield while still leaving 30–50 kg of residual N per hectare can be highly beneficial for the following crop and the margins for the producer. On the other side, even a partial amount of N accumulated within the leaves if translocated and loaded into grain could help to increase yield potential or improve grain quality. Ostmeyer et al. (2022) outlined the advantages and disadvantages of the stay-green trait in grain sorghum. The authors stated how under optimal conditions (adequate water and N), a hybrid with increased terminal senescence that has a higher efficiency of translocating N to the grain would be more productive than that of a stay-green hybrid. This is evident in this study, where a higher yield could have been obtained with more optimized stay-green trait, that is, a senescent hybrid.

5 | CONCLUSIONS

Our findings indicate that higher rate and split application of N plays a role in increasing grain yield and protein in the tested sorghum hybrid. Split application of N at earlier growth stage at panicle initiation proved to be beneficial compared to the late application of N at booting. Utilizing high-throughput phenotyping has the capability of predicting grain yield and protein content and can be a good tool to be used in sorghum-breeding programs. Intra-panicle grain dynamics indicate a possibility to increase yield and quality by targeting the grains at the bottom portion of the panicle. In addition, when growing a strong stay-green hybrid, it appears that the split application of N has no effect on NUE, unlike environment. The large proportion of N taken up and accumulated in the leaves at harvest (RLN) provides an opportunity for rendering sorghum to be a highly sustainable crop through efficient N cycling in a cropping system or by breeding better translocating and N use efficient sorghum hybrids. Currently, the impacts of in-season split application of N under different water available conditions are being investigated on multiple commercially available grain sorghum hybrids to confirm the key findings and to recommend agronomic and breeding approaches for increased nitrogen use efficient and sustainable sorghum for the future.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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