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Regulation of Density and Fertilization on Crude Protein Synthesis in Forage Maize in a Semiarid Rain-Fed Area

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Abstract: Density and fertilization mode are the key factors regulating crude protein synthesis in forage maize; however, there is a lack of systematic understanding of the regulation mechanism. Here, the nitrogen/potassium ratio (N/K), free amino acid (AA) content, crude protein synthesis key enzyme activities (nitrate reductase (NR), glutamine synthetase (GS) and glutamic pyruvic transaminase (GPT)) and crude protein content and yield in leaves, stems, and grain of forage maize, as well as the relationships among them, were explored. The results showed that the N/K of DL-40 (60,000 plants per ha^{-1}, 40% N topdressing at large trumpet stage) and DH-50 (75,000 plants per ha^{-1}, 50% N topdressing at large trumpet stage) significantly increased in leaves, stems, and grain, and correspondingly, NR, GS, and GPT activities in leaves, NR activities in stems, NR and GPT activities in grain, and delayed the decline in AA content. After tasseling, for DL-40 and DH-50 the crude protein content increased by 74.1% and 39.8% in leaves, respectively, 19.9% and 25.6% in grain, respectively, and crude protein yield increased by 16.7% and 35.2% in leaves, respectively, and 23.5% and 25.9% in grain, respectively. There were significant quadratic parabolic relationships of NR, GS, and GPT activities with proportion of topdressing. There was a significant relationship of crude protein content with NR activity in leaves, with NR, GS, and GPT activities in stems and with GPT activity in grain. Regulating the key enzyme activity by adjusting the density and fertilization can significantly improve the crude protein yield of forage maize. Treatments DL-40 and DH-50 significantly increased crude protein content and yield by increasing plant N/K, NR activity in leaves, NR activity in stems, and GPT activity in grain, but slowed the decrease in AA content in leaves, stems, and grain.

Keywords: forage maize quality; density; fertilization methods; crude protein synthesis; regulation; quality improvement

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1. Introduction

The arid region of the northwest Loess Plateau is an important farming–pastoral zone and a key area of grain for forage in China. Recently, the planting area of forage maize has increased year by year, and low crude protein content is the main problem affecting its quality [1,2], which restricts the sustainable and efficient development of the herbivorous livestock industry. Improving the crude protein content is crucial to improving forage conversion efficiency and livestock breeding benefits [3,4]. Plant density and fertilizer are the two most critical factors regulating crude protein synthesis of forage maize [5–7].

There are many reports on the effects of plant density and fertilization on crude protein content of forage maize. It is believed that with increased planting density, plant height, stem diameter, leaf number, leaf area per plant, dry matter, and crude protein content per plant decrease significantly [8–10], but the population leaf area index increases, and the total biomass initially increases and then decreases [11].

The effect of nitrogen (N) application rate on the crude protein content of forage maize is controversial. With increased N application rate, some studies have shown that crude protein content increases [5], but others suggest that it initially increases and then decreases [12] and that postponing N application can significantly increase crude protein content [13]. Studies on the regulation of crude protein synthesis suggest that N, as an important component of enzymes and nucleic acids, is the source of regulation of protein accumulation and regulates protein post-translational modification [14,15]. Plant potassium (K) content significantly affects N metabolism-related enzyme activity, which is closely related to N metabolism processes [16,17]. Adjusting the N/K of plants by regulating N application rate can significantly regulate nitrate reductase (NR), glutamine synthetase (GS), and glutamic pyruvic transaminase (GPT) activities and the content of free amino acids (AAs), promote the transformation rate of AAs to grain and directly determine the protein accumulation rate.

The above studies were conducted on the grain or leaves of crops, and mainly focus on phenomena, and less on mechanism. It can be seen that there is a lack of systematic research on the physiological regulation mechanism of density and fertilization on the growth and crude protein content of forage maize, especially in the special production system of double ridge and furrow plastic mulching (PM) and furrow planting. The purpose of this study was to clarify the physiological regulation mechanism of the increase in crude protein content from the perspective of "plant N/K–substrate concentration–enzyme activity–crude protein synthesis" in a dry farming area with mulching. This work can enrich and develop theoretical knowledge regarding forage maize quality cultivation, and play a positive role in promoting the management of planting structure in this area.

2. Materials and Methods

2.1. Site Description and the Experimental Treatments

This study site was located at the Dingxi Experimental Station of the Gansu Academy of Agricultural Sciences. The station is located in the northwest Loess Plateau (Anding District, Dingxi, Gansu Province, 104°36′ E, 35°35′ N) at an altitude of approximately 1970 m. The soil water content (*w*/*w*) at field capacity and wilting point was 23 and 7.2%, respectively. Based on 35 years of records (1986–2020), mean annual rainfall average is 415 mm with nearly 68% occurring during June–September, the relative variability of the precipitation is 24% and mean annual temperature is 6.2 °C. Average annual sunshine hours are 2500 h. The soil is a light loam of loess origin, without salinity and alkalinity problems. The soil nutrient status of soil organic matter, total nitrogen (N), total phosphorus, total potassium, ammonium-N, nitrate-N, available phosphorus, and available potassium was 11.99 g kg^{-1}, 1.16 g kg^{-1}, 0.73 g kg^{-1}, 17.28 g kg^{-1}, 4.8 mg kg^{-1}, 0.8 mg kg^{-1}, 8.66 mg kg^{-1}, and 121.50 mg kg^{-1}, respectively.

2.2. Precipitation and Air Temperature

The precipitation and air temperature data were obtained from meteorological data measured at the Dingxi Experimental Station of the Gansu Academy of Agricultural Sciences(Tangjiabao Village, Unity Town, Anding District, Dingxi City, Gansu Province, 104°36′ E, 35°35′ N), the annual rainfall of the experimental area in 2019 was 516.8 mm and rainfall during the growth period of forage maize was 358.8 mm; the corresponding values in 2020 were 518.9 and 386.2 mm, with both years considered to be wet. The annual average temperature of 2019 was 7.2 °C and average temperature of the crop growth period was 15.9 °C, with corresponding values of 6.9 and 15.6 °C in 2020.

2.3. Experimental Design

Eight treatments were applied during the maize growing seasons (mid-April to late September) of 2019–2020. All treatments had double ridges and furrows with PM, Nitrogen fertilizer was used in ordinary urea (nitrogen content 46%). Treatments received 300 kg N ha^{-1}, 150 kg P_2O_5 ha^{-1}, and 90 kg K_2O ha^{-1}. We applied two densities

(60,000 and 75,000 plants per ha^{-1}) and four fertilization methods (all basal application and top dressing 40%, 50%, and 60% at large trumpet stage). There were eight treatments: (1) full basal fertilizer (CK-L); (2) 40% N topdressing at large trumpet stage (DL-40); (3) 50% N topdressing at large trumpet stage (DL-50); (4) 60% N topdressing at large trumpet stage (DL-60), all at a density of 60,000 plants per ha^{-1}; (5) full basal fertilizer (CK-H); (6) 40% N topdressing at large trumpet stage (DH-40); (7) 50% N topdressing at large trumpet stage (DH-50); and (8) 60% N topdressing at large trumpet stage (DH-60), all at a density of 75,000 plants per ha^{-1}.

The method of double ridges and furrows with PM (Figure 1) comprised a wide ridge 10 cm high and 70 cm wide, and a narrow ridge 15 cm high and 40 cm wide, both of which had PM applied. Maize was sown in the furrows. The experiment was laid out in a randomized complete block design, with four replications per treatment. The area of each plot was 63 m^2 (7 m × 9 m). Maize was sown at 5–8 cm depth in the furrows with a soil water content of 10–15% in the 0–30 cm soil layer using a maize planting machine. The spacing was 30/24 cm at the planting densities of 60,000/75,000 plants per ha^{-1}. Maize was sown around 20 April and harvested around 8 September each year.

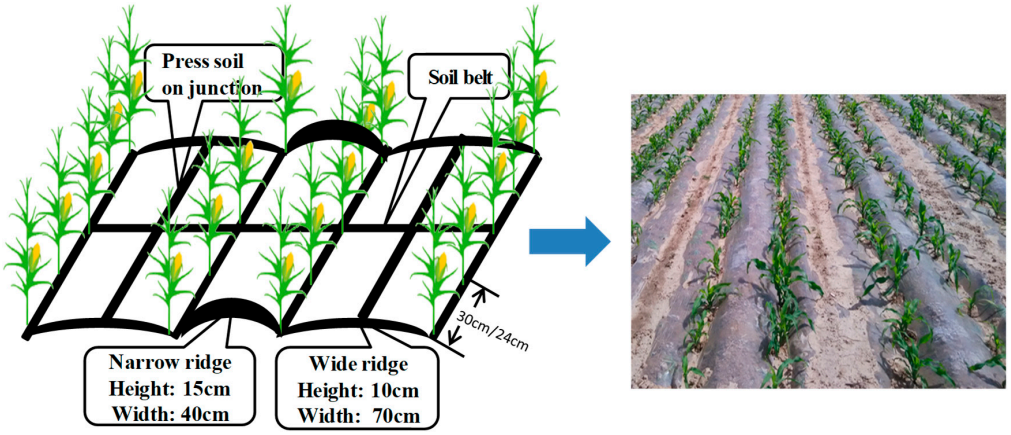

Figure 1. Schematic diagram of PM. Note: The left diagram is a schematic diagram, the right diagram is a field planting diagram, and the middle arrow only indicates that the right diagram is the actual effect of the left diagram.

2.4. Plant Total N and K Content Measurements

The samples were dried for 30 min at 105 °C to fix the chlorophyll and then dried at 80 °C to constant weight and crushed. The total N was determined by Kjeldahl method, and total K was determined by flame photometer (Beijing zhongke keer instrument company limited, Beijing, China. M425) after samples were passed through a 40-mesh sieve.

2.5. AA Content Measurements

A small amount of 80% ethanol was added in a mortar to 3 g of sample, which was then ground to a homogenate, moved to a 100 mL volumetric flask and filtered. Solids were obtained from 20 mL of filtrate by vacuum evaporation. The solids were dissolved in distilled water to prepare 0.1 g mL^{-1} of diluent and AAs were determined by ninhydrin colorimetry.

2.6. NR Activity Measurements

First, 1.0 g of fresh material and 10 mL of phosphate buffer (0.1 mol L^{-1}, pH 7.5) were placed into a test tube, and placed in darkness for 20 min. After removal, 1 mL of 30% trichloroacetic acid was added and then immediately oscillated. Of the extract, 2 mL was placed in a test tube, 4 mL of 1% sulfonamide solution and 4 mL of 0.02% α-naphthylamine solution were added, followed by shaking and standing for 35 min, after which the absorbance was measured at 520 nm.

2.7. GS Activity Measurements

A sample, weighing 0.5 g fresh weight was ground in an ice bath with 0.05 mol/L Tris-HCl buffer extract 5 mL (containing 2 mmol L^{-1} $MgSO_4$, 2 mmol L^{-1} DTT and 0.4 mol L^{-1} sucrose at pH 8.0) and centrifuged for 20 min at 15,000× *g* and 4 °C, producing a supernatant containing the crude extract of GS. The optical density (OD) value at 540 nm was measured by UV-4802S spectrophotometer and GS activity was expressed as formation of γ-glutamylhydroxamate at 37 °C.

2.8. GPT Activity Measurements

A sample of 0.5 g fresh weight was ground in an ice bath with 0.05 mol/L Tris-HCl buffer extract 5 mL (containing 0.2 mol L^{-1} trimethylolaminomethane (50 mL) and 0.2 mol L^{-1} HCl (44.2 mL) diluted with distilled water to 200 mL at pH 7.2) and centrifuged for 20 min at 20,000× *g* and 4 °C. The supernatant contained the crude enzyme extract. The optical density (OD) value at 505 nm was measured by UV-4802S spectrophotometer and GPT activity was expressed as the catalytic production of 1 nmol pyruvate per minute.

2.9. Crude Protein Content Measurements

The samples were placed in an electric blast drying oven (Beijing hongda tianju test equipment company limited, Beijing, China. DHG-9070A), dried at 105 °C to fix the chlorophyll and then dried at 80 °C to constant weight and crushed. After passing through a 40-mesh sieve, crude protein was determined by the Kjeldahl method.

2.10. Statistical Analysis

All data were subjected to analysis of variance (SAS Institute, Cary, NC, USA; 2004), and differences between means were tested by the least significant difference test (Duncan multiple comparison method, $p < 0.05$).

3. Results

3.1. Effects of Density and Fertilization on N/K in Forage Maize Organs

Density and fertilization method had significant effects on N/K in different organs of forage maize (Figure 2). Compared with CK-L, the N/K in DL-40 treatment increased by 13.3% in leaves, 37.0% in stems after tasseling, and 71.0% in grain, which were all significant changes. The N/K in leaves, stems and grain was increased by topdressing at high plant density. Among treatments, DH-50 increased the most and compared with CK-H, the N/K significantly increased by 43.7% in leaves, 83.9% in stems, and 68.9% in grain.

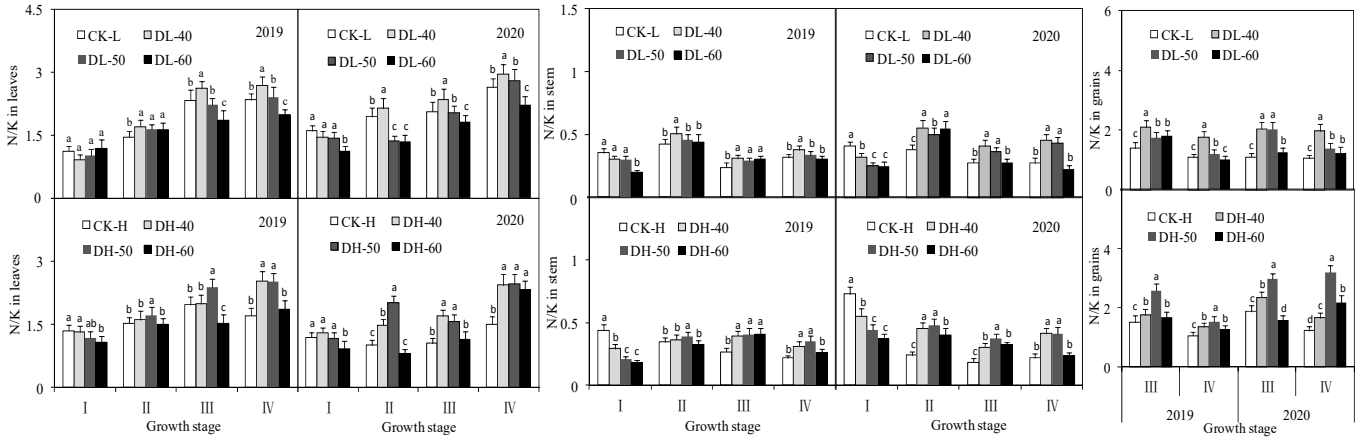

Figure 2. Effects of density and fertilization on N/K in organs of forage maize. Notes: I: jointing stage; II: tasseling stage; III: filling stage; IV: dough stage, the same notation applies to the other figures. Different letters on bars indicate significant differences among treatments at the 0.05 probability level.

3.2. Effects of Density and Fertilization on AA in Forage Maize Organs

The AA content in leaves and stems decreased gradually with the growth period, and topdressing did not change the trend, but did hinder the decline of AA (Figure 3). The AA content of CK-L, DL-40, DL-50, and DL-60 decreased by 55.8–80.1%, 46.3–74.3%, 55.7–85.6%, and 52.9–89.8% in leaves at the jointing–dough stage, respectively. The AA content of CK-H, DH-40, DH-50, and DH-60 decreased by 76.4–79.2%, 54.6–81.8%, 48.5–72.9%, and 54.5–74.3% in leaves at the jointing–dough stage, respectively. The AA content was lower in stems than in leaves and decreased less in DL-40 and DH-50 than other treatments. The AA content in grain was higher than in leaves and stems, and was significantly higher in DL-40 and DH-50 than in other treatments.

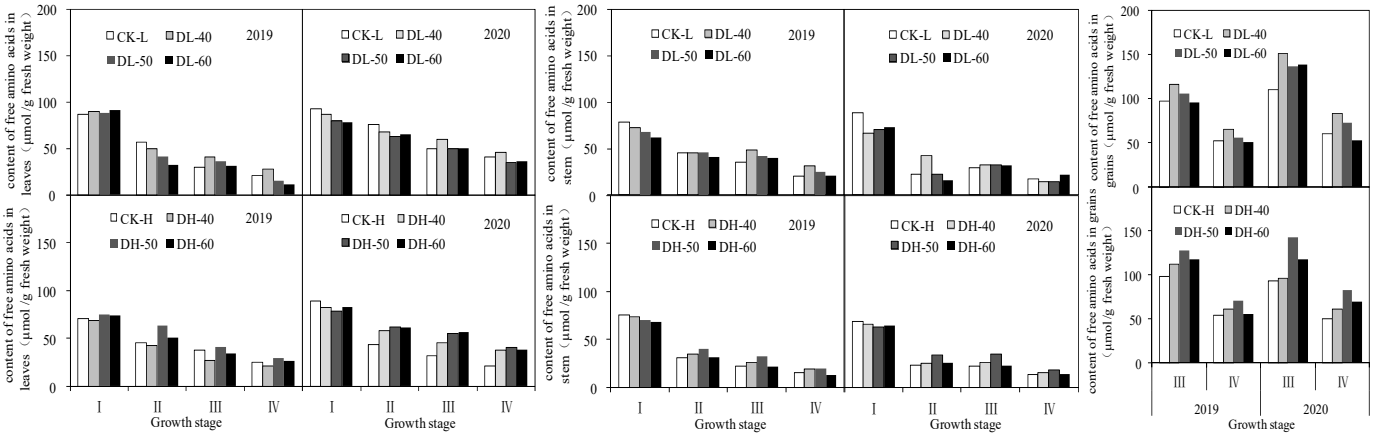

Figure 3. Effects of density and fertilization on AA content in organs of forage maize.

3.3. Effects of Density and Fertilization on Enzyme Activities in Forage Maize Organs

The effects of density and fertilization methods on key enzyme activities in different organs differed (Figure 4). Enzyme NR is a N assimilation enzyme and a rate-limiting enzyme for inorganic N metabolism. Treatments DL-40 and DH-50 had significantly higher NR activity in leaves, stems, and grain compared with other treatments. The NR activity in DL-40 increased by 14.3% in leaves, 34.8% in stems, and 30.1% in grain compared with CK-L, respectively, and corresponding increases for DH-50 of 41.05%, 53.17%, and 47.11% compared with CK-H.

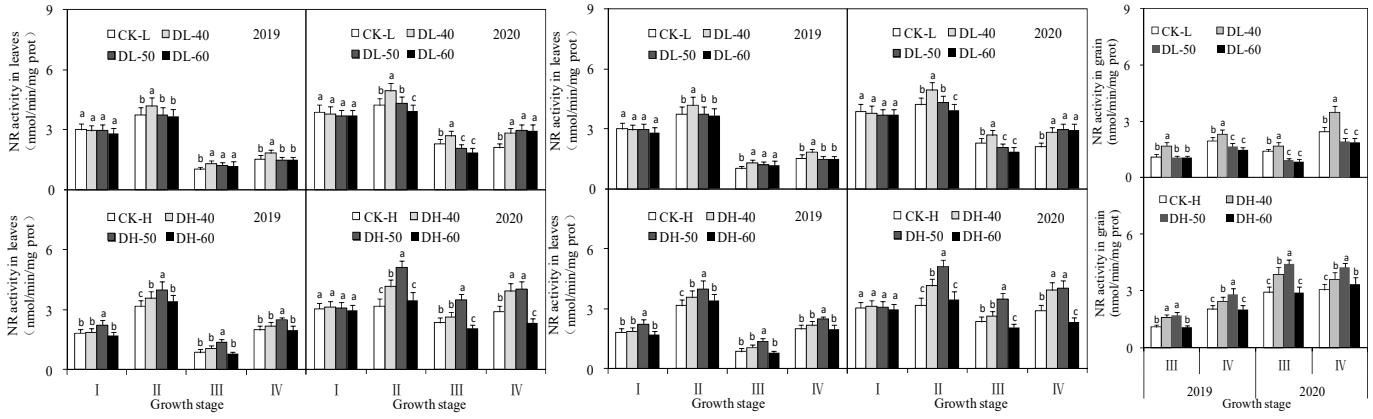

Figure 4. Effects of density and fertilization on NR activity in organs of forage maize. Note: Different letters on bars indicate significant differences among treatments at the 0.05 probability level.

Enzyme GS is the center of N assimilation, and its activity directly affects plant N assimilation and recycling ability (Figure 5). The GS activity of maize leaves initially increased and then decreased, with the maximum at tasseling stage. Treatments DL-40 and

DH-50 had significantly higher GS activity in leaves at tasseling and filling stages, with DL-40 increasing by 24.6–48.7% and 34.8–58.4% compared with CK-L, respectively, and corresponding increases for DH-50 of 25.1–115.0% and 69.0–106.3% compared with CK-H, respectively. Density and fertilization method had no significant effect on GS activity of stems and grain.

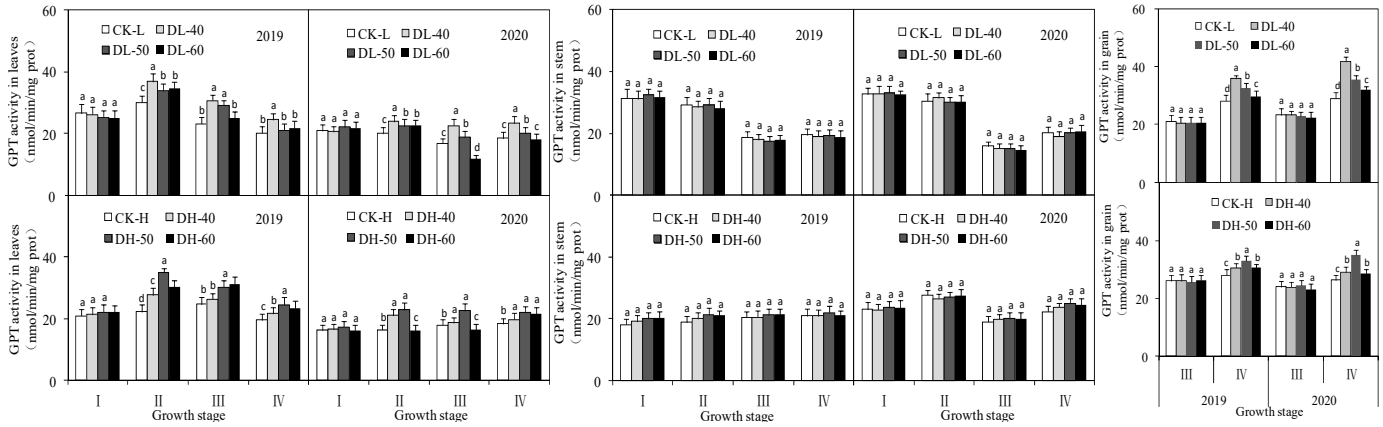

Figure 5. Effects of density and fertilization on GS activity in organs of forage maize. Note: Different letters on bars indicate significant differences among treatments at the 0.05 probability level.

An increase in GPT activity is beneficial to the balanced supply of AA substrate for protein synthesis, and reduces the limitations of individual AAs for protein synthesis (Figure 6). Compared with CK-L, DL-40 and DL-50 had significantly higher GPT activity in leaves at the tasseling and filling stages, and DL-40 increased by 29.8–33.7% and 21.8–26.2%, respectively, with corresponding increases for DL-50 of 11.6–23.2% and 4.5–8.2%. Density and fertilization had no significant effect on GPT activity in stems. Topdressing significantly raised the GPT activity of grain at the dough stage, with increases for DL-40 and DH-50 of 23.8–43.8% and 10.0–32.8% compared with CK-L and CK-H, respectively.

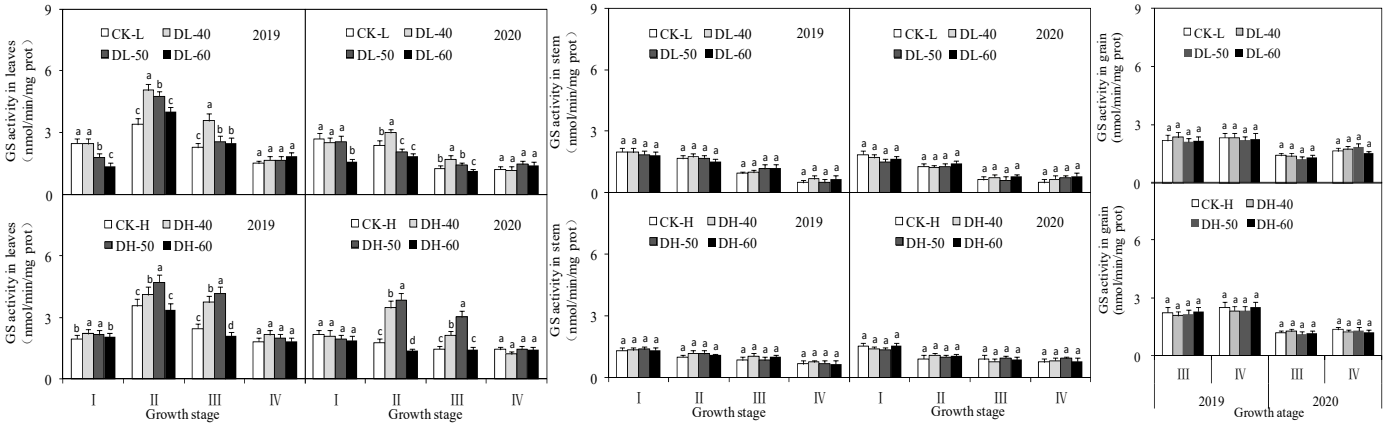

Figure 6. Effects of density and fertilization on GPT activity in organs of forage maize. Note: Different letters on bars indicate significant differences among treatments at the 0.05 probability level.

3.4. Effects of Density and Fertilization on Protein Content and Yield in Forage Maize Organs

The crude protein content in organs was ranked in the order of leaf > grain > stem (Figure 7). Compared with CK-L, at the tasseling and filling stages, the crude protein content of leaves significantly increased by 81.9% and 66.4%, respectively, with corresponding increases for crude protein yield of 34.9% and 22.0%, respectively, in DL-40 (Figure 8). Compared with CK-H, DH-40 and DH-50 significantly increased crude protein content and yield in leaves at the tasseling, filling and dough stages, with increases in content of 38.3%,

37.9%, and 41.6%, respectively, and correspondingly in yield of 48.1%, 22.3%, and 30.9% in DH-50. Density and topdressing had no significant effect on crude protein content of stems. Compared with CK-L, the crude protein content in grain increased by 24.4% and 15.3% and the crude protein yield increased by 16.7% and 30.3% in DL-40 at the filling and dough stages, respectively. The crude protein content of grain in DH-50 was 27.2% and 24.0%, respectively, higher than that of CK-H and crude protein yield was 20.4% and 31.3% higher at the filling and dough stages, respectively.

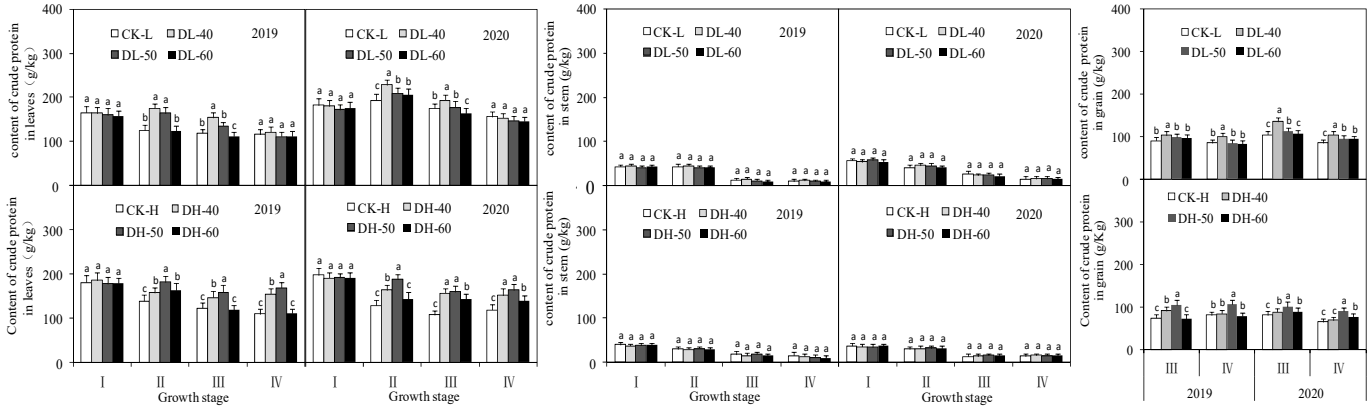

Figure 7. Effects of density and fertilization on the crude protein content in organs of forage maize. Note: Different letters on bars indicate significant differences among treatments at the 0.05 probability level.

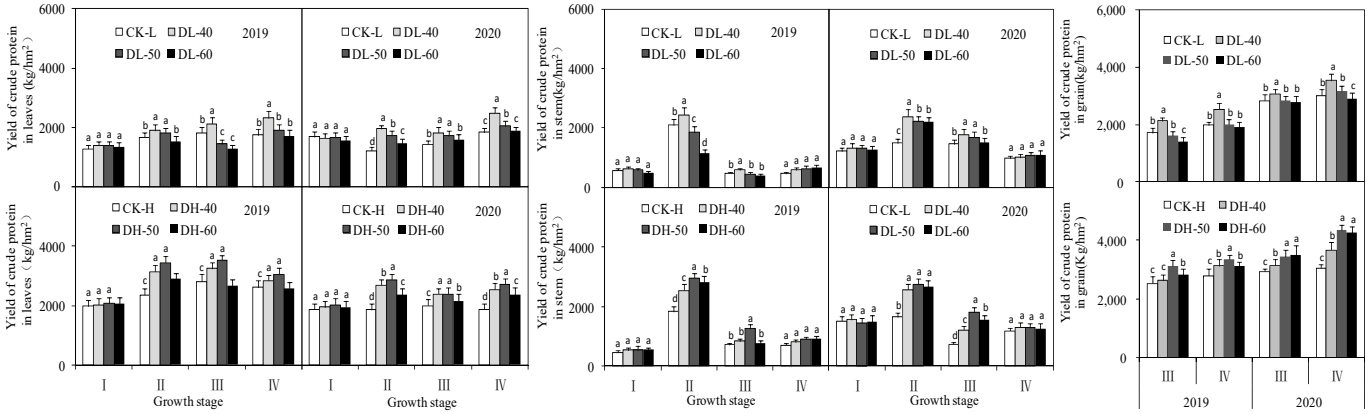

Figure 8. Effects of density and fertilization on the crude protein yield in organs of forage maize. Note: Different letters on bars indicate significant differences among treatments at the 0.05 probability level.

With the increase in density, the ratio of leaf biomass and total biomass was basically the same, the ratio of stem biomass decreased by 4.9 %, and the ration of grain biomass increased by 3.34 % (Table 1).

3.5. Relationships of Key Enzyme Activities with Density and Fertilization Method

The NR and GS activities of forage maize had no significant relationships with planting density. There was a significant linear negative relationship between GPT activity and planting density (R^2 = 0.0641, $p < 0.05$, Figure 9). The activities of NR, GS, and GPT all showed significant quadratic parabolic relationships with the proportion of topdressing (R^2 = 0.0674, 0.0753, and 0.0666, respectively, all $p < 0.05$). With the increase in proportion of topdressing, the activities of these enzymes first increased and then decreased (Figure 10). Under the experimental conditions, the NR and GS activities of forage maize

were only regulated by fertilization, while GPT activity was regulated by both density and fertilization.

Table 1. Average yield of biomass (fresh-weight yield, kg·hm^{-2}) and the yield component during 2019–2020.

Treatment	Total Biomass (kg·hm^{-2})	Leaf Biomass (kg·hm^{-2})	Stem Biomass (kg·hm^{-2})	Grain Biomass (kg·hm^{-2})	Ratio of Leaf Biomass and Total Biomass	Ratio of Stem Biomass and Total Biomass	Ratio of Grain Biomass and Total Biomass
CK-L	93,771	18,865	49,382	25,523	20.12	52.66	27.22
DL-40	96,111	17,779	48,700	29,632	18.50	50.67	30.83
DL-50	91,142	14,849	50,179	26,114	16.29	55.06	28.65
DL-60	82,228	14,333	47,526	20,368	17.43	57.80	24.77
CK-H	97,582	22,149	45,260	30,173	22.70	46.38	30.92
DH-40	92,625	18,404	43,446	30,774	19.87	46.91	33.22
DH-50	99,483	18,987	54,192	26,305	19.09	54.47	26.44
DH-60	79,925	13,531	39,019	27,376	16.93	48.82	34.25

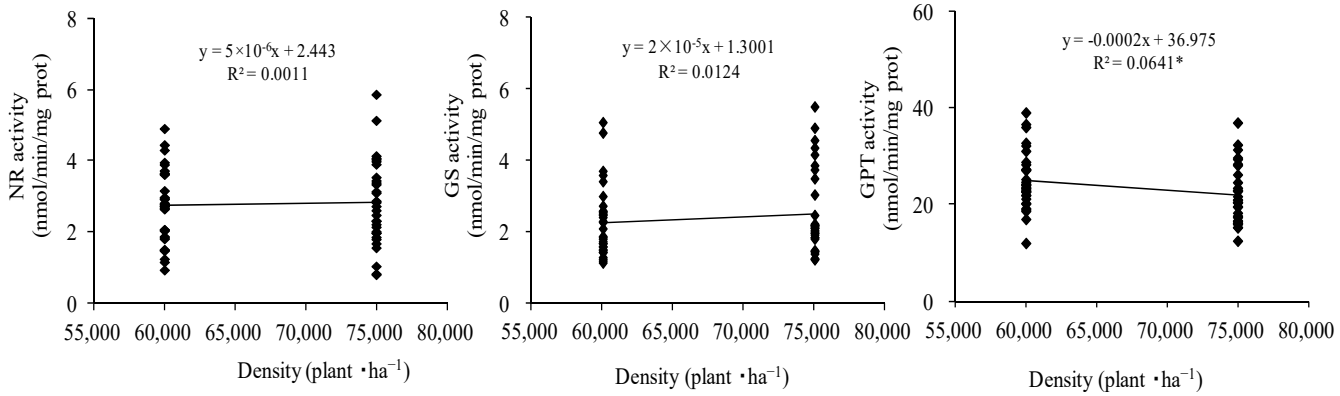

Figure 9. Relationships between plant density and key enzyme activity. Note: "*" indicate a significant correlation at the 0.05 probability level. The dots represent the measured values, and the lines represent the correlation trend in the figure.

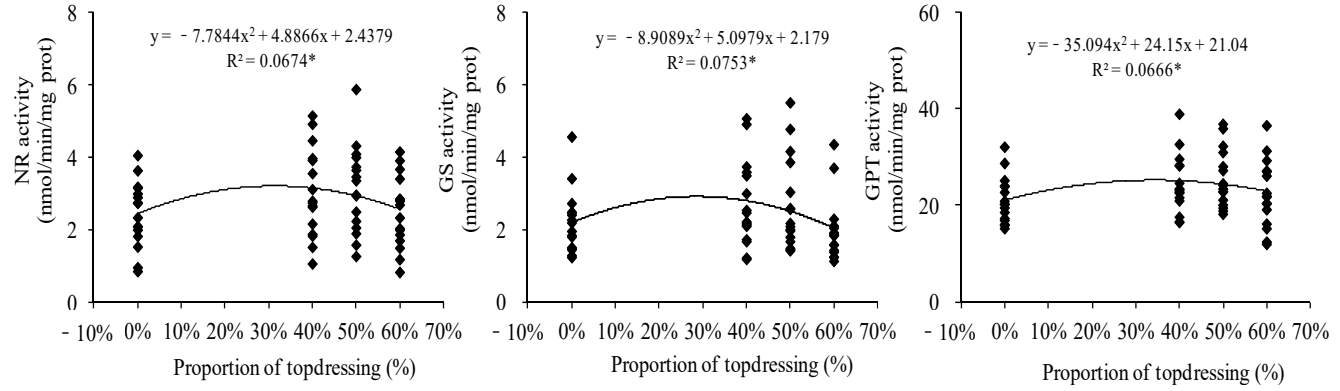

Figure 10. Relationships between fertilization and key enzyme activity. Note: "*" indicate a significant correlation at the 0.05 probability level. The dots represent the measured values, and the lines represent the correlation trend in the figure.

3.6. Relationships between Crude Protein Content and Key Enzyme Activities

The relationships between crude protein content and key enzyme activities differed for the different organs (Figures 11–13). There was no significant relationship between crude protein content and GS and GPT activities in leaves, but there was an extremely significant

quadratic parabolic relationship with NR activity in leaves (R^2 = 0.1112, $p < 0.01$). The crude protein content of stem was significantly related to NR, GS, and GPT activities (R^2 = 0.074, $p < 0.05$; 0.7394, $p < 0.01$; and 0.5726, $p < 0.01$, respectively). The crude protein content of grain was significantly positively related to GPT activity in grain (R^2 = 0.2195, $p < 0.01$).

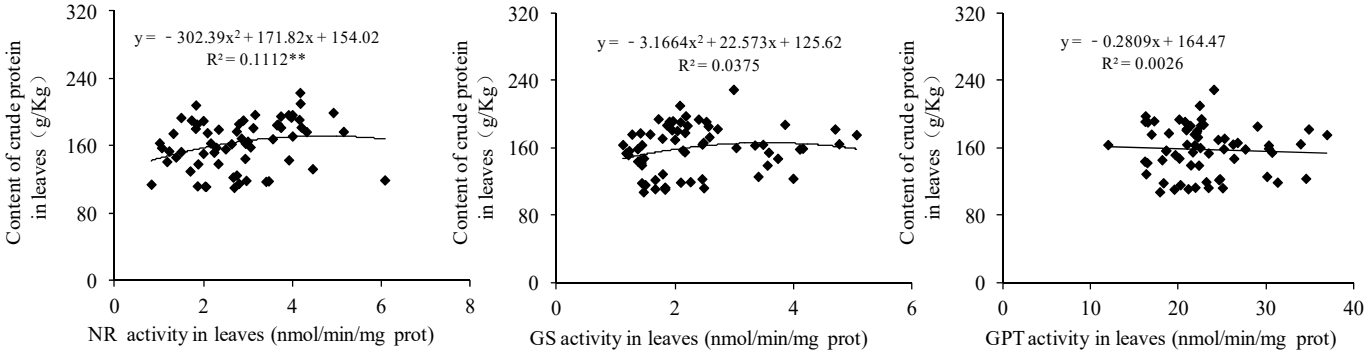

Figure 11. Relationships between crude protein content and key enzyme activity in leaves. Note: "**" indicate a significant correlation at the 0.01 probability level. The dots represent the measured values, and the lines represent the correlation trend in the figure.

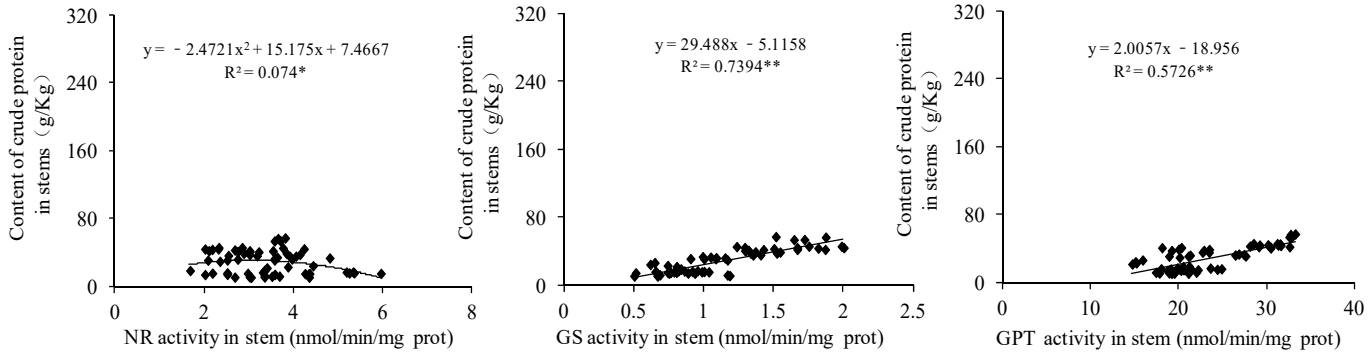

Figure 12. Relationships between crude protein content and key enzyme activity in stems. Note: "*" and "**" indicate the significant correlation at the 0.05 and 0.01 probability level, respectively. The dots represent the measured values, and the lines represent the correlation trend in the figure.

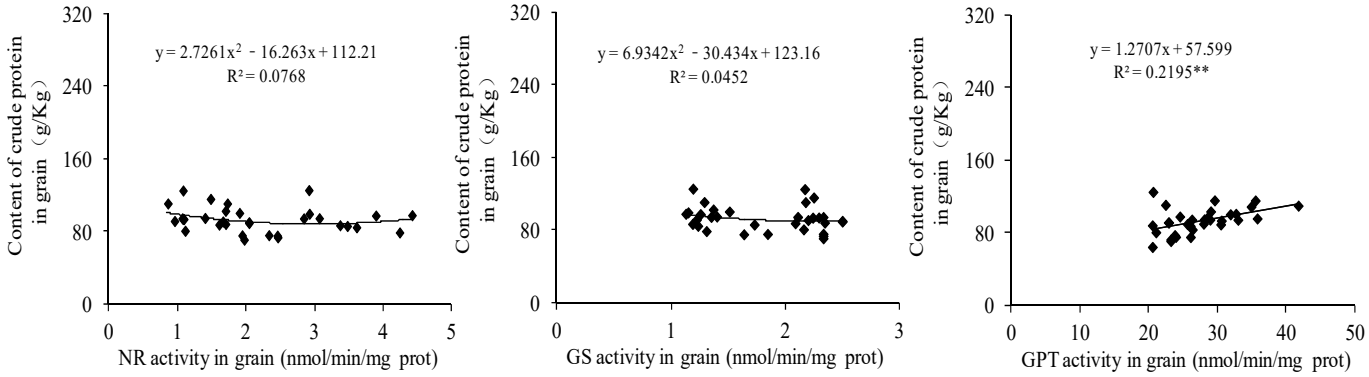

Figure 13. Relationships between crude protein content and key enzyme activity in grain. Note: "**" indicate a significant correlation at the 0.01 probability level. The dots represent the measured values, and the lines represent the correlation trend in the figure.

3.7. Relationships of Crude Protein Yield with Density and Fertilization Methods

Crude protein yield of leaves and grain showed very significant quadratic parabolic relationships with fertilization method for 60,000 plants per ha^{-1}, with an R^2 of 0.2918 and 0.2379, respectively (Figures 14 and 15), but there was no significant relationship for

stems. For 75,000 plants per ha^{-1}, leaves and grain crude protein yield and fertilization methods showed significant quadratic parabolic relationships (R^2 = 0.2285, $p < 0.01$ and 0.1688, $p < 0.05$, respectively) but there was no significant relationship for stems.

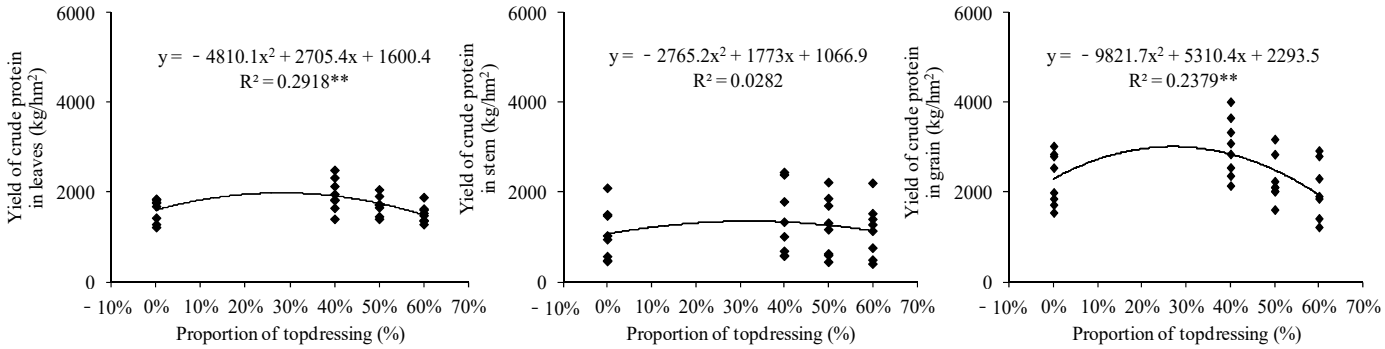

Figure 14. Relationship between crude protein yield and fertilization method for 60,000 plants per ha^{-1}. Note: "**" indicate a significant correlation at the 0.01 probability level. The dots represent the measured values, and the lines represent the correlation trend in the figure.

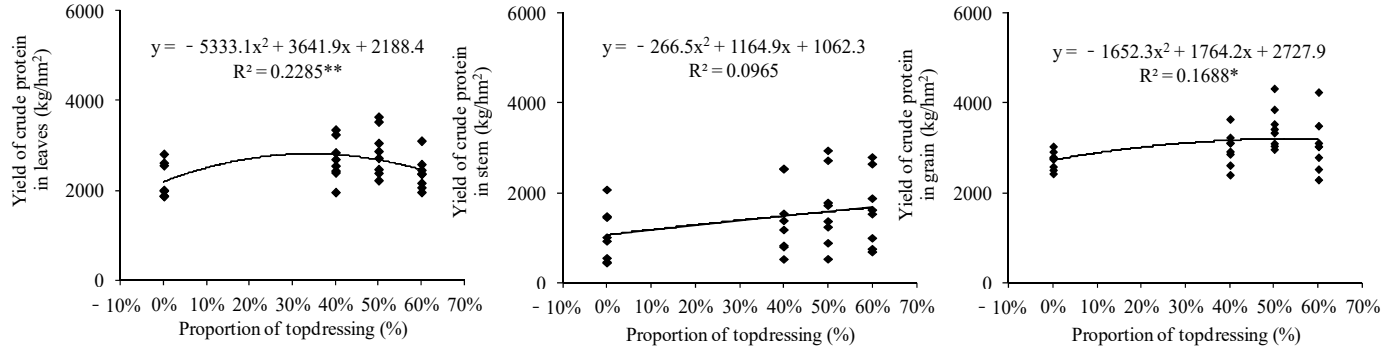

Figure 15. Relationship between crude protein yield and fertilization method for 75,000 plants per ha^{-1}. Note: "*" and "**" indicate the significant correlation at the 0.05 and 0.01 probability level, respectively. The dots represent the measured values, and the lines represent the correlation trend in the figure.

4. Discussion

4.1. N Application and Density Significantly Regulate Protein Synthesis in Forage Maize

Protein synthesis speed can be regulated at the transcription and translation levels. As an important component of enzymes and nucleic acids, N mainly affects protein synthesis at the translation level [12]. Nitrogen management can significantly regulate plant N/K, the activities of key enzymes in protein synthesis (e.g., NR, GS, and GPT), the content of free AAs which is the immediate form of protein synthesis substrate, promote the transformation rate of AAs to grain and then regulate protein synthesis and its yield [15–18]. In this study, N fertilizer application methods had significant effects on N/K in different organs of forage maize.

Treatments DL-40 and DH-50 significantly increased N/K of maize leaves, stems, and grain from the tasseling stage onward, but the change trend differed among organs, and regulation of key enzyme activities also differed (Figures 2 and 3). After topdressing, for leaves, the N/K was the highest at dough stage, and the activities of NR, GS, and GPT were highest at tasseling stage in DL-40 and DH-50. For stems, N/K was highest at tasseling stage, NR activity was highest at dough stage and GS and GPT activities did not change significantly. The N/K of grain was higher at filling than at dough stage, but the three enzyme activities were all lower than that at dough stage. Thus, N/K significantly regulated NR, GS, and GPT activities in leaves, NR activity in stems, and NR and GPT activities in grain. Treatments DL-40 and DH-50 regulated plant N/K by regulating N

fertilizer application. Appropriate N/K increased the activity of key enzymes of protein synthesis, enhanced the assimilation and recycling ability of plant N [14], slowed the decline of free Aas [15] (Figure 2), ensured a balanced supply of AAs for protein synthesis, and reduced the limitation of individual AA deficiencies on protein synthesis [19,20]. Therefore, after topdressing, the crude protein content of leaves in DL-40 and DH-50 increased by 66.35–81.9% and 37.9–41.6%, respectively, and crude protein yield increased by 22.0–34.9% and 22.3–48.1%. The view that N fertilizer management can significantly regulate protein synthesis is consistent with previous studies [15–18,21]. However, most existing studies have focused on N application rate and few addressed N fertilization methods under equal N conditions [21,22]. The studies for different basal/topdressing and topdressing stages agreed that "nitrogen fertilizer postponing can significantly improve the crude protein content of forage maize" [23,24]. The same results were obtained in this paper, but proportion of topdressing and period most conducive to synthesis of crude protein in forage maize needs further study.

Most studies showed that, plant density increased within a certain range, forage maize yield increased but crude protein content decreased [9,25]. Excessive density leads to inappropriate resource allocation and yield reduction. The main reason is that high density increases intraspecific competition for available soil nutrients, water, and light [26–28], resulting in less N uptake and lower quality. In addition, plant cells cannot fully expand, resulting in increased cell wall thickness at high densities [29] and decreased nutritional value. Forage maize needs an appropriate planting density to balance yield and quality [30]. Some studies have suggested that density had no significant effect on forage-fresh weight yield and crude protein content of the whole plant [6,31]. Thus, the effect of planting density on crude protein content of forage maize is controversial. In this study, plant densities of 60,000 and 75,000 plants per ha^{-1} were set. With the increase in density, the forage-fresh weight yield increased (Table 1) and crude protein content in leaves and stems decreased, but were not significantly affected in grain. The crude protein content in the whole plant decreased, consistent with existing research [9,25]. However, the crude protein yield of the whole plant increased significantly, which is critical for forage maize, and there are few related reports.

4.2. Regulation of Fertilization Method and Density on Protein Synthesis Enzymes

The level of N metabolism is an important indicator of capacity for crude protein synthesis and yield formation, and enzyme activity is an extremely important factor for the N metabolism process. Activities of NR, GS, and GPT are closely related to protein synthesis, and are significantly regulated by N fertilizer application rate, type, and application period [14,18,25,32]. Overall, the activities of key enzymes in N metabolism increased with the increase in N nutrition level, and ultimately increased the grain protein content and yield [33]. However, when the N application rate exceeded a certain level, activities of these enzymes decreased.

Studies have shown that increasing N application rate can significantly improve NR activity in wheat and maize leaves, and NR and GS activities initially increase and then decrease [34,35], and postponing application of N fertilizer can significantly increase the NR and GS activities of peanut at the late growth stage [13]. In this study, with the increase of the proportion of topdressing, the NR, GS, and GPT activities initially increased and then decreased, consistent with existing research [13,35].

The effect of planting density on N metabolism enzyme activity is controversial. On the one hand, with the increase in planting density, enzyme activity in N metabolism decreases [36]. On the other hand, high planting density can promote accumulation of N in wheat grain, enhance the activity of N assimilation enzymes, and improve the grain protein content [37,38]. In this study, the NR and GS activities of forage maize had no significant relationship with planting density, and GPT activity was negatively related with planting density, which is inconsistent with existing research. The possible reasons are different crop characteristics and different planting patterns and regions, which needs further study.

4.3. Regulation of Density and Fertilization Methods on Protein Content and Yield

Increasing N fertilizer can provide more energy and electrons for AA synthesis, improve the activity of key enzymes for protein synthesis [33] and the conversion rate of Aas [22], and the protein contents of grain significantly improved for wheat and peanut [37,39]. However, excessive N supply leads to low C/N ratio and vigorous leaf N metabolism leads to large consumption of photosynthate, as well as a reduced output rate of photosynthate, resulting in feedback inhibition of photosynthate on photosynthetic organs, and then reduced crop yield and quality. In addition, different basal application ratios affect protein synthesis by regulating NR and GS activities [13]. Appropriately increasing the planting density can compensate for the lack of biomass per plant and increase total biomass. However, excessive density leads to weakening of crop canopy light-transmission, shortening of leaf functional period, and premature senescence of leaves, thereby reducing crop photosynthetic capacity, N absorption, and protein content [35,40] (Li et al., 2018; Wei et al., 2015).

In this study, the effects of different density and proportions of topdressing on the activities of key enzymes, crude protein content and yield in different organs, and whole plants of forage maize were studied. The density and fertilization method affected crude protein content and yield by regulating the activities of key enzymes. Density was not significantly related with NR and GS activities, but was significantly negatively related with GPT activity. Grain crude protein content was significantly positively related with GPT activity (Figure 9). With the increased proportion of topdressing, the activities of the three enzymes initially increased and then decreased (Figure 10). The crude protein content was significantly related with GS activity in leaves, with NR, GS, and GPT activities in stems, and with GPT activity in grain. Therefore, the crude protein content in leaves and grain in DL-40 and DH-50 was higher than in other treatments, but the difference was not significant in stems (Figures 11–13). Topdressing ratio also affected crop growth (Figures 14 and 15), and the variation trends of crude protein content and yield differed. The crude protein yields of leaves and grain were significantly positively related with density, and the crude protein yields of leaves and grain increased with increased plant density. The proportion of topdressing showed significant regulation of crude protein yield of leaves and grain but not stems.

5. Conclusions

Plant density and fertilization had significant regulatory effects on plant N/K, crude protein synthesis enzymes activity, and crude protein content and yield. With increasing density, GPT activity decreased significantly. With the increased proportion of topdressing, the NR, GS, and GPT activities initially increased and then decreased. There were significant relationships between crude protein content and GS activity in leaves, NR, GS, and GPT activities in stems and GPT activity in grain. Both leaf and grain crude protein yields had a significant quadratic parabolic relationship with fertilization method. Treatments DL-40 and DH-50 significantly increased N/K of all organs of forage maize, GS activity in leaves, NR activity in stems, and GPT activity in grain, and slowed the decrease in AA content in plants. Under the conditions of higher enzyme activity and sufficient substrate concentration, the crude protein yield of DL-40 and DH-50 increased significantly by 16.7% and 35.2% in leaves, respectively, and correspondingly by 23.5% and 25.9% in grain. It can be seen that DL-40 (60,000 plants per ha^{-1}, 40% N topdressing at large trumpet stage) and DH-50 (75,000 plants per ha^{-1}, 50% N topdressing at large trumpet stage) can be widely used as planting methods for improving the quality of forage maize.

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References

1. Li, J.; Li, L.L.; Zhang, L.J.; Chen, L.L.; Xie, J.H. Effects of nitrogen application on yield and forage quality of grain and forage maize. *Pratac. Sci.* **2015**, *32*, 442–449. [CrossRef]
2. Lal, M.; Singh, D.; Dass, S. Heterosis studies for yield and qulity traits in rabi quality protein maize. *Agric. Sci. Digest.* **2011**, *31*, 206–210.
3. Apple, J.K.; Maxwell, C.V.; Bass, B.E.; Yancey, J.W.S.; Payne, R.L.; Thomson, J. Effects of reducing dietary crude protein levels and replacement with crystalline amino acids on growth performance, carcass composition, and fresh pork quality of finishing pigs fed ractopamine hydrochloride. *J. Anim. Sci.* **2017**, *95*, 4971–4985. [CrossRef]
4. Yang, T.Y.; Ma, X.Y.; Gong, X.X.; Peng, C.; Huang, J.; Zhao, G.Q.; Zhan, K. Effects of different protein levels on gluconeogenesis of Holstein bull calves. *J. China Agric. Univ.* **2022**, *27*, 187–194. [CrossRef]
5. Lamptey, S.; Yeboah, S.; Li, L. Response of Maize Forage Yield and Quality to Nitrogen Fertilization and Harvest Time in Semi−arid Northwest China. *Asian J. Res. Agric. For.* **2018**, *1*, 1–10. [CrossRef]
6. Salama, H.S.A. Yield and nutritive value of maize (*Zea mays* L.) forage as affected by plant density, sowing date and age at harvest. *Ital. J. Agron.* **2019**, *14*, 114–122. [CrossRef]
7. Sun, J.Y.; Gao, J.L.; Wang, Z.G.; Yu, X.F.; Hu, S.P.; Liu, J.; Bao, H.Z.; Huo, X.X.; Bai, Y.L.; Yan, D. Effect of planting density on forage yield and nutritive value of different maize warieties. *Acta Agrestia Sin.* **2019**, *27*, 1733–1742. [CrossRef]
8. Ferreira, G.; Teets, C.L. Effect of planting density on yield, nutritional quality, and ruminal in vitro digestibility of corn for silage grown under on-farm conditions. *Prof. Anim. Sci.* **2017**, *33*, 420–425. [CrossRef]
9. Wang, X.J.; He, H.J.; Kou, S.R.; Zhou, Y.Q.; Liu, Z.X.; Yang, Y.Z.; Lian, X.R.; Zhou, W.Q. Effect of different planting densities on biomass yield and quality for various varieties of silage maize. *Pratac. Sci.* **2019**, *36*, 169–177. [CrossRef]
10. Jia, M.Y.; Yao, Z.Y.; Li, C.Q.; Wang, L.; Wang, J.Q.; Liu, G.H.; Yang, J.G. Effect of planting density on growth, yield and quality of silage maize. *Feed Res.* **2020**, *43*, 105–108. [CrossRef]
11. Di, S.X.; Su, D.S.; Zhu, Y. Effects of different planting densities on yield and nutritive value of silage corn. *China Feed.* **2018**, *12*, 26–29. [CrossRef]
12. Zhang, Q.; Zhang, L.; Cui, R.Q.; Liu, Q.; Du, J.; Cao, G.Y.; Tian, X.P. Effect of nitrogen fertilizer on yield and forage quality of silage maize. *J. Tianjin Agric. Univ.* **2020**, *27*, 1–6. [CrossRef]
13. Yao, R.; Zhao, K.N.; Xie, C.; Song, H.L.; Xu, S.; Yu, H.Q.; Zhang, Z.; Wang, J.; Jiang, C.J.; Zhao, S.L.; et al. Effects of postponing nitrogen application on nitrogen enzyme activities, nitrogen utilization efficiency and yield in peanut. *Chin. J. Oil Crop Sci.* **2022**, *44*, 877–883. [CrossRef]
14. Zhong, Y.; Yang, M.; Cai, J.; Wang, X.; Zhou, Q.; Cao, W.; Dai, T.; Jiang, D. Nitrogen topdressing timing influences the spatial distribution patterns of protein components and quality traits of flours from different pearling fractions of wheat (*Triticum aestivum* L.) grains. *Field Crops Res.* **2018**, *216*, 120–128. [CrossRef]
15. Troise, A.D.; Wiltafsky, M.; Fogliano, V.; Vitaglione, P. The quantification of free Amadori compounds and amino acids allows to model the bound Maillard reaction products formation in soybean products. *Food Chem.* **2018**, *247*, 29–38. [CrossRef] [PubMed]
16. Ou, X.H.; Cui, X.M.; Zhu, D.W.; Guo, L.P.; Liu, D.H.; Yang, Y. Lowering Nitrogen and Increasing Potassium Application Level Can Improve the Yield and Quality of Panax notoginseng. *Front. Plant Sci.* **2020**, *11*, 1–14. [CrossRef]
17. Liu, X.W. Effect of Soil Potassium Deficiency on Potassium Absorption, Quality and Carbon and Nitrogen Metabolism of Different Winter Wheat Varieties. Master's Thesis, Henbei Agricultural University, Baoding, China, 2020.
18. Xiang, F.; Li, W.; Liu, H.Y.; Zhou, L.Y.; Yin, X.; Zeng, Z.X. Effects of nitrogen levels on key enzyme activities and non-structural carbohydrates in nitrogen metabolism in tea leaves. *Acta Ecol. Sin.* **2019**, *39*, 9052–9057. [CrossRef]
19. Balotf, S.; Kavoosi, G.; Kholdebarin, B. Nitrate reductase, nitrite reductase, glutamine synthetase, and glutamate synthase expression and activity in response to different nitrogen sources in nitrogen-starved wheat seedlings. *Biotechnol. Appl. Biochem.* **2016**, *63*, 220–229. [CrossRef] [PubMed]
20. Yusuf, A.; Deepa, P. Influence of N nutrients on GS activity and putative ammonium transporter1;2 (SaAMT1;2) expression in sandal plants (*Santalum album* L.). *Trees* **2017**, *31*, 1773–1784. [CrossRef]
21. Zhang, M.; Ma, D.; Ma, G.; Wang, C.; Xie, X.; Kang, G. Responses of glutamine synthetase activity and gene expression to nitrogen levels in winter wheat cultivars with different grain protein content. *J. Cereal Sci.* **2017**, *74*, 187–193. [CrossRef]

22. Zhen, S.; Deng, X.; Zhang, M.; Zhu, G.; Lv, D.; Wang, Y.; Zhu, D.; Yan, Y. Comparative Phosphoproteomic Analysis under High-Nitrogen Fertilizer Reveals Central Phosphoproteins Promoting Wheat Grain Starch and Protein Synthesis. *Front. Plant Sci.* **2017**, *8*, 67. [CrossRef] [PubMed]
23. Xu, M.Y.; Xie, F.; Li, Y.Q.; Li, J.G.; Yin, X.; Cao, Y.F. Effects of Fertilizing on Nutritional Quality and Feeding Value of maize silage. *Chin. J. Anim. Nutr.* **2011**, *23*, 1043–1051. [CrossRef]
24. Uzun, S.; Özaktan, H.; Uzun, O. Effects of Different Nitrogen Dose and Sources as Top-Dressing on Yield and Silage Quality Attributes of Silage Maize. *An. Acad. Bras. Ciênc.* **2020**, *92* (Suppl. S1). [CrossRef] [PubMed]
25. Zhao, M.; Feng, Y.; Shi, Y.; Shen, H.; Hu, H.; Luo, Y.; Xu, L.; Kang, J.; Xing, A.; Wang, S.; et al. Yield and quality properties of silage maize and their influencing factors in China. *Sci. China Life Sci.* **2023**, *65*, 1655–1666. [CrossRef]
26. Mandic, V.; Krnjaja, V.; Bijelic, Z.; Tomic, Z.; Simic, A.; Stanojkovic, A.; Petricevic, M.; Caro-Petrovic, V. The effect of crop density on yield of forage maize. *Biotehnol. Stoc.* **2018**, *31*, 567–575. [CrossRef]
27. Zhang, P.L.; Guo, T.W.; Liu, X.W.; Li, S.T.; Zeng, J.; Qin, X.L.; Dong, B. Effect of plant density and nitrogen application rate on yield, nitrogen and water use efficiencies of spring maize under whole plastic-film mulching and double-furrow sowing. *Plant Nutr. Fert. Sci.* **2019**, *25*, 579–590. [CrossRef]
28. Meng, Y.; Liu, Z.Y.; Li, J.; Gu, W.R.; Wei, S. Effects of nitrogen rate on grain yield and quality and key enzyme metabolism of spring maize under high density. *Southwest China J. Agric. Sci.* **2020**, *33*, 1146–1152. [CrossRef]
29. Lukuyu, B.; Murdoch, A.; Romney, D.; Mwangi, D.; Njuguna, J.; McLeod, A.; Jama, A. Integrated maize management options to improve forage yield and quality on smallholder farms in Kenya. *Field Crop. Res.* **2013**, *153*, 70–78. [CrossRef]
30. Morris, T.F.; Murrell, T.S.; Beegle, D.B.; Camberato, J.J.; Ferguson, R.B.; Grove, J.; Ketterings, Q.; Kyveryga, P.M.; Laboski, C.A.M.; McGrath, J.M.; et al. Strengths and Limitations of Nitrogen Rate Recommendations for Corn and Opportunities for Improvement. *Agron. J.* **2018**, *110*, 1–37. [CrossRef]
31. Ferreira, G.; Alfonso, M.; Depino, S.; Alessandri, E. Effect of planting density on nutritional quality of green-chopped corn for silage. *J. Dairy Sci.* **2014**, *97*, 5918–5921. [CrossRef]
32. Wang, S.-Y.; Liu, Q.; Shi, Y.-X.; Li, H. Effects of potassium on nitrogen translocation and distribution and nitrogen metabolism enzyme activities of sweet potato. *Ying Yong Sheng Tai Xue Bao = J. Appl. Ecol.* **2016**, *27*, 3569–3576.
33. Demir, Z.; Keçeci, M.; Tunç, A.E. Effects of nitrogen fertigation on yield, quality components, water use efficiency and nitrogen use efficiency of silage maize (*Zea mays* L.) as the second crop. *J. Plant Nutr.* **2021**, *44*, 373–394. [CrossRef]
34. Zeng, M.; Xie, J.H.; Li, L.L.; Zechariah, E.; Liu, X.N.; Du, C.L.; Wang, J. Response of physilolgical and bilchemical indexes of flag leaf and grain filling of rainfed spring wheat to long-term nitrogen application rates. *J. Gansu Agric. Univ.* **2021**, *56*, 47–55. [CrossRef]
35. Li, W.L.; Lv, Y.J.; Liu, X.M.; Tong, T.; Cao, X.B.; Gu, W.R.; Wei, S. Effects of nitrogen fertilizer on nitrogen metabolism enzymes, nitrogen use and yield of maize with different nitrogen efficiency. *Southwest China J. Agric. Sci.* **2018**, *31*, 1829–1835. [CrossRef]
36. Li, R.F.; Liu, P.; Yang, Q.L.; Ren, H.; Dong, S.T.; Zhang, J.W.; Zhao, B. Effects of lower leaf senescence on carbon and nitrogen distribution and yield formation in maize (*Zea mays* L.) with high planting density. *Acta Agron. Sin.* **2018**, *44*, 1032–1042. [CrossRef]
37. Wang, J.F.; Wang, Z.Z.; Gu, F.X.; Mou, H.M.; Wang, Y.; Duan, J.Z.; Feng, W.; Wang, Y.; Guo, T. Effects of nitrogen fertilizer and plant density on carbon metabolism, nitrogen metabolism and grain yield of two winter wheat varieties. *Sci. Agric. Sin.* **2021**, *54*, 4070–4083. [CrossRef]
38. Lv, L.H.; Tao, H.B.; Wang, P.; Liu, M.; Zhao, M.; Wang, R.Z. Carbon and nitrogen metabolism and nitrogen use efficiency in summer maize under different planting densities. *Acta Agron. Sin.* **2008**, *34*, 718–723. [CrossRef]
39. Li, D.; Zhang, D.; Wang, H.; Li, H.; Fang, Q.; Li, H.; Li, R. Optimized Planting Density Maintains High Wheat Yield Under Limiting Irrigation in North China Plain. *Int. J. Plant Prod.* **2019**, *14*, 107–117. [CrossRef]
40. Wei, S.; Wang, X.; Zhang, J.; Liu, P.; Zhao, B.; Li, G.; Dong, S. The role of nitrogen in leaf senescence of summer maize and analysis of underlying mechanisms using comparative proteomics. *Plant Sci.* **2015**, *233*, 72–81. [CrossRef]