



Article

The Combined Application of Urea and Fulvic Acid Solution Improved Maize Carbon and Nitrogen Metabolism

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Abstract: It has been reported that fulvic acid (FA) application improves soil structure and nutrient availability. However, the effects of combined application of urea (U) and FA solution on the photosynthesis and nitrogen metabolism in maize (*Zea mays* L.) have rarely been reported. In this study, pot experiments were conducted in 2017 and 2018, and the effects of combined application of urea and FA solution (U+FA) on soil available nutrient contents, maize endogenous hormone concentrations, carbon and nitrogen metabolism-related enzyme concentrations, maize yield, and nitrogen use efficiency (NUE) were researched. Compared with the U treatment, the maize yield and NUE in the U+FA treatment were significantly increased by 8.31% and 17.09 percentage points in 2017 and by 16.90% and 24.31 percentage points in 2018. At the jointing and 12-leaf (V12) stages of maize, soil NH_4^+ content increased by 139.32% and 12.08%, separately, in the U+FA treatment. At the V12 stage, the auxin, nitrate reductase, nitrite reductase, and glutamine synthetase concentrations in maize root were increased by 42.31%, 74.17%, 16.61%, and 45.60%, respectively, and the concentrations of pyruvate phosphate dikinase and phosphoenolpyruvate carboxylase in maize leave were increased by 29.40% and 42.96%, respectively, in the U+FA treatment. The combined application of urea and FA solution significantly improved soil nutrient availability, increased the concentrations of endogenous hormones in maize, stimulated the activities of enzymes related to nitrogen metabolism, promoted the photosynthetic carbon assimilation efficiency, and ultimately improved crop yield and NUE.

Keywords: fulvic acid; maize yield; endogenous hormone; nitrogen metabolism-related enzyme



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1. Introduction

Maize (*Zea mays* L.) is a nicotinamide adenine dinucleotide phosphate malic enzyme (NADP-ME) type C_4 plant [1]. As one of the most important food crops, maize has a larger production area than rice in China. With the development of intensification agricultural systems, maize production is becoming increasingly important. The North China Plain (NCP) is a major grain-producing region in China. It contributes 31% of the total maize yield, where it accounts for 32% of the total maize planting area in China [2]. However, soil nutrient supply generally cannot meet the demand of maize during its growth. The element nitrogen (N) is essential for the syntheses of genetic materials, metabolic enzymes, hormones, and proteins in plants. It has a strong influence on the growth, development, yield, and quality of crops [3]. In order to achieve high maize yield and quality, chemical fertilizers, especially N fertilizers, are commonly applied to soils. During the summer maize growing season, many observed N fertilizers have been observed, which can be as high

300–360 kg N ha⁻¹ yr⁻¹ [4–6], which surpasses the crop requirements of approximately 200 kg N ha⁻¹ yr⁻¹ [7–9]. The disproportionate use of N fertilizer results in substantial waste of resources, increased costs and decreased economic benefits, all of which seriously hinder the region's agricultural development. As a result, the new fertilization methods and nitrogen use efficiency should be studied with great efforts during the summer maize growth period.

Urea (46% N) has the highest N content of all inorganic N fertilizers and contributes to approximately 40% of total N fertilizer capacity in China. At 20–25 °C, urea applied to the soil would be hydrolyzed by microorganisms and urease secreted by plant root into ammonium bicarbonate within 5–7 days, and the produced NH₄⁺ ions would be absorbed by plants for growth [10,11]. Those NH₄⁺ ions that are not absorbed by plants would be adsorbed by the negatively charged soil colloids or oxidized to NO₂⁻ by ammonia-oxidizing bacteria and then to NO₃⁻ by nitrifying bacteria [12]. They are readily carried away by runoff into the surface waters or leached into the subsurface soil layers and then the underground water, causing eutrophication of waters. Under anaerobic conditions, NO₃⁻ would be reduced to NO, N₂O, and N₂ by bacteria and fungi, causing emissions of greenhouse gases [13]. Therefore, excessive urea application would not only bring environmental concerns, but also lead to low nitrogen use efficiency (NUE). It is reported that the average NUE of summer maize over is only 25–30% in the NCP, which is far lower than that in developed countries [9]. Hence, improving NUE, reducing environmental pollution and greenhouse gas emissions caused by excessive fertilization, and developing efficient and sustainable and intensive agricultural have become the focus of food production.

Integrating inorganic fertilizer with organic amendments is an effective strategy for ensuring the sustainability of agricultural system [14,15]. Fulvic acid (FA) is a widely used amendment in soils environment that has been proved to be beneficial in promoting plant growth and increasing crop yields [16,17]. According to the extraction source of fulvic acid, it can be divided into natural and biochemical fulvic acid. Natural fulvic acid is a kind of macromolecule organic weak acid mixture formed by a series of decomposition and transformation of animal and plant remains under microbial, geophysical and chemical action, which can be extracted from soil, water and coal [18–20]. Biochemical fulvic acid is a type of weakly acidic organic matter extracted from agricultural waste by microbial fermentation technology or straw-based ammonium sulfite pulping process [21]. Its chemical properties are similar to those of natural fulvic acid [22,23]. This is attributed to various functional groups of biochemical fulvic substance including carboxylic acid. Previous research has discovered that fulvic acid is the end result of plant lignin breakdown into compounds [24]. However, due to the obvious involvement of multiple impurities, poor availability, and ease of transformation in soil, most plant polyphenols are restricted to the experimental stage, and they are not considered suitable for promotion in crop production practice. Fulvic acid, which is isolated from certain plant polyphenols, contains a mixture of soluble organic acids formed by the polymerization of chemically and biologically active functional groups, influences N cycling, and has a high potential for promoting crop growth and influencing the succedent validity of urea. Some studies found that the phenolic hydroxyl and quinone groups in FA could undergo redox reaction with urease sulfhydryl to form FA-urease complexes, thereby inhibiting urease activity and delaying the conversion and decomposition of urea [25,26]. In addition, the functional groups (carboxyl and phenolic hydroxyl) in FA compete with phosphorus (P) for the adsorption sites on mineral surface, reducing P fixation and increasing P availability [27–29]. Researchers found that FA application increased the mitotic sites of maize lateral root and improved the H⁺-ATPase activity in root plasma membrane, which stimulated root growth and enhanced the nutrient uptake ability of maize [30,31]. In addition, FA was found to directly up-regulate the nitrate uptake and assimilation genes in maize root, promoting nitrate absorption and enhancing N assimilation efficiency [32].

In this study, pot experiments were conducted in 2017 and 2018 to evaluate the synergistic effects of urea in combination with FA solution on soil nutrient availability,

carbon and nitrogen metabolism, and endogenous hormone concentrations of maize at the physiological level. This study aims to: (1) investigate the interactions between the urea and FA solution applications and their effects on soil nutrients and NUE; (2) understand the roles of nitrogen metabolism-related enzyme, endogenous hormones and photosynthesis in widening of maize production when urea and FA solution are applied together; and (3) determine the factors that influence maize yield and NUE. The findings are expected to provide a reference for combined application of urea and FA so as to reduce the risk of environmental pollution and improve fertilizer use efficiency and crop yield.

2. Materials and Methods

2.1. Experimental Materials

The soil utilized was classified as Typic Hapludalf [33] or Typic-Hapli-Udic Argosols [34] and was obtained from a depth of 0–20 cm in the experimental base of the Science and Innovation Park of Shandong Agricultural University. At the same time, the fundamental properties of soil were shown in Table 1.

Table 1. The fundamental properties of soil in this experimental site.

pH (1:2.5)	Organic Matter Content	Total N	Nitrate N	Ammonium N	Available Phosphorus	Available Potassium
	(g kg ⁻¹)				(mg kg ⁻¹)	
7.85	12.01	0.65	72.35	9.44	13.22	92.22

The FA of the biochemical type used was extracted from crop straw and supplied by Shandong Quanlin Jiayou Fertilizer Co. Ltd., Liaocheng, China. It had a pH of 5.40, a N content of 2%, and a K₂O content of 3%. The fertilizers, including urea (46% N), potassium chloride (60% K₂O), diammonium phosphate (18% N, 46% P₂O₅), and calcium superphosphate (46% P₂O₅) were purchased from local market.

2.2. Experimental Design and Management

The pot experiments were conducted at the National Engineering Research Center for Efficient Utilization of Soil and Fertilizer Resources, Shandong Agricultural University, Taian, Shandong Province, China (36°9′40″ N, 117°9′48″ E). The experimental location has a moderate continental monsoon climate, with an annual average temperature of 13.2 °C and an annual average precipitation of 401.77 mm.

In each ceramic pot (30 cm in diameter and 36 cm in height), approximately 20 kg soil was added, with 1 kg sand at the bottom. Three treatments were set up with four replicates: (1) no nitrogen fertilizer (CK); (2) application of urea (U); (3) combined application of urea and FA solution (U+FA). The fertilizers and FA were applied at 225 kg N·ha⁻¹, 150 kg P₂O₅·ha⁻¹, 75 kg K₂O·ha⁻¹, and 45 kg FA·ha⁻¹. The quantity of fertilizer used in the pot experiment was doubled, resulting in 4.0 g N·pot⁻¹, 2.6 g P₂O₅·pot⁻¹, 1.4 g K₂O·pot⁻¹, and 5.4 g FA pot⁻¹ [16,17]. These fertilizer rates were calculated based on the common practices in the area. The P and K fertilizers were applied once as basal fertilizer in all treatments, while nitrogen was equally split-applied as basal fertilizer and topdressing (at the jointing stage) in the U and U+FA treatments. In 2017, maize (*Zea mays* L. ‘zhengdan 958’) was sown on June 20.

During the experiment, an automatic drip irrigation system was used to keep the soil water content at 70 ± 5% of field capacity in each pot [35,36]. Weeding and pest management were carried out in accordance with local producers’ best practices. The experiment was repeated in 2018, with sowing and harvesting at the same dates as in 2017.

2.3. Sampling and Chemical Analyses

The functional groups of FA were determined by Fourier Transform Infrared with waves number ranging from 400 cm⁻¹ to 4000 cm⁻¹ (Nicolet Nexus 410, thermo nicole, ...

America). The spectrometer had a resolution of 2 cm^{-1} , signal-to-noise ratio of 50,000:1 and 32 scanning times (Supplementary Figure S1).

Soil inorganic nitrogen (NH_4^+ -N and NO_3^- -N) was extracted with 0.01 M CaCl_2 at soil to water ratio of 1:10 and quantified using a continuous flow injection analyzer (AA3-A001-02E, Bran-Luebbe, Germany). Soil available phosphorus was assessed by 0.5 M NaHCO_3 (pH 8.5) and colorimetrically quantified using the molybdenum antimony method [37]. Flame photometry was used to assess available potassium, which was extracted with 1.0 M of $\text{CH}_3\text{COONH}_4$ at a pH of 7.0 [38].

At the seedling (July 9), jointing (July 20), 12-leaf (V12, August 13), and silking stages (September 11) of maize, plant height and stem diameter (between the second and third nodes from the bottom) were measured, and soil samples were gathered from the 0–20 cm layer in each pot for available nutrient analysis. At the jointing stage, the relative concentration of chlorophyll (i.e., SPAD value) was measured on the third completely grown leaf from the top using a chlorophyll meter (SPAD-502, Minolta, Japan). Net photosynthetic rate (P_n), stomatal conductance (G_s), and transpiration rate (T_r) were monitored on the ninth leaf from the top using a Li-6400XT portable photosynthesis system (Licor Inc., Lincoln, NE, USA). In addition, the second fully expanded leaf from the top (i.e., the largest functional leaf) was collected from each plant, washed with deionized water, snap frozen in liquid nitrogen, and stored at $-80\text{ }^\circ\text{C}$ before being analyzed for endogenous hormones and carbon and nitrogen metabolism-related enzyme activities. At the V12 stage, leaves were collected again for analyses of carbon and nitrogen metabolism-related enzyme activities. In addition, root was sampled, washed with deionized water, snap frozen in liquid nitrogen, and stored at $-80\text{ }^\circ\text{C}$ before analyses for endogenous hormones and nitrogen metabolism-related enzyme activities. At September 29, maize was harvested. After ears per plant, rows per ear, grains per row, and 100-grain weight were recorded, plant samples were oven-dried first at $105\text{ }^\circ\text{C}$ for 15 min and then at $65\text{ }^\circ\text{C}$ to constant weight.

The concentrations of endogenous hormones, including auxin (IAA), gibberellin (GA), abscisic acid (ABA), and zeatin nucleoside (ZR), and the activities of carbon and nitrogen metabolism-related enzymes, including glutamine synthetase (GS), glutathione reductase (GSR), nitrate reductase (NR), glutamate dehydrogenase (GLDH), nitrite reductase (NiR), glutamate synthase (Fd-GOGAT), pyruvate phosphate dikinase (PPDK), and phosphoenolpyruvate carboxylase (PEPC), in root and leave were estimated using the ELISA kit (Shanghai HengYuan Biological Technology Co. Ltd., Shanghai, China). An ELISA kit uses a double-antibody sandwich method to determine the concentrations of various enzymes in samples. The purified enzyme capture antibody was placed on a microplate to make a solid phase antibody. To form the antibody-antigen enzyme-labeled antibody complex, the enzyme was applied to the coated micropores one at a time and then combined with the detecting antibody-labeled using the enzyme labeled reagent horseradish peroxidase (HRP) to form antibody antigen enzyme labeled antibody complex. After complete washing, add the base material (TMB) for color development. TMB is transformed to blue by HRP enzyme catalysis, and then to yellow via acid action. The color depth was shown to be positively associated with enzyme content in the sample. Using enzyme labeling equipment, measure the absorbance [optical density (OD) value] at 450 nm and use the standard curve to calculate the enzyme concentration in the sample [16,39].

2.4. Data Analyses

The following equation was used for the calculation of NUE [35]:

$$\text{NUE (\%)} = ((\text{TN} - \text{TN}_0)/\text{SN}) \times 100 \quad (1)$$

where TN is maize total N uptake in the U or U+FA treatment, TN_0 is maize total N uptake in CK, and SN is total N from fertilizer in the U or U+FA treatment.

Data were processed using Microsoft Excel 2019 and analyzed by ANOVA using IBM SPSS Statistics 24 (SPSS Inc., Armonk, New York, USA). Means were separated using SNK's test (Student–Newman–Keuls) ($p < 0.05$). The Mantel test were performed using the vegan

library in R to determine correlations between maize yield as well as endogenous hormones and carbon and nitrogen metabolism-related enzyme activities at the Physiological level. The principal component analysis (PCA) was used to investigate determinants on the application of different fertilizations in impacting maize yield and NUE [40]. Figures were plotted using SigmaPlot 2004 (Version 12.0, Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Maize Yield and NUE

The combined application of urea and FA solution had a positive effect on maize grain yield and the yield components (Table 2). Compared to U treatment, the yield and aboveground dry biomass of maize in the U+FA treatment were significantly increased by 8.31% and 5.80%, respectively, in 2017 and by 16.90% and 15.84%, respectively, in 2018. Meanwhile, the rows per ear in the U+FA treatment was significantly higher by 18.90% in 2017. The NUE in the U+FA treatment was higher by 17.09 and 24.31 percentage points in 2017 and 2018, respectively. The results showed that combining urea and FA solution not only increased maize grain yield but also improved NUE as compared with the application of urea only.

Table 2. Maize aboveground dry biomass, yield and yield components, and nitrogen use efficiency (NUE) in different fertilization treatments.

Treatment	Aboveground Dry Biomass (g pot ^{−1})	100-Grain Weight (g)	Grains per Row	Rows per Ear	Grain Yield (g pot ^{−1})	Yield Increase (%)	NUE (%)
2017							
CK	83.3 c	25.4 b	13.0 a	12.0 c	36.1 c	−78.92	-
U	289.9 b	29.5 ab	14.5 a	32.8 b	171.3 b	-	36.96
U+FA	306.7 a	30.4 a	14.5 a	39.0 a	185.5 a	8.31	54.05
2018							
CK	162.1 c	21.0 b	12.0 b	25.0 b	85.2 c	−56.14	-
U	328.9 b	28.8 a	14.0 ab	36.7 a	194.2 b	-	24.70
U+FA	381.0 a	30.7 a	15.3 a	40.3 a	227.0 a	16.90	49.01

Treatments: no nitrogen fertilizer (CK); application of urea (U); combined application of urea and fulvic acid (U+FA). Values within a same column followed by different letters are significantly different between treatments for a same year according to least significant difference test ($p < 0.05$).

3.2. Soil Available Nutrients

Compared to the U treatment at the jointing, V12, and silking stages, soil $\text{NH}_4^+\text{-N}$ content of the U+FA treatment was significantly increased by 139.32%, 12.08%, and 392.93%, respectively (Table 3). As FA was dissolved to irrigate maize root, the $\text{NO}_3^-\text{-N}$ level of soil was significantly improved by 12.28%, compared with the U treatment at V12 stage. At the seedling stage, soil available phosphorus content of the U+FA treatment was significantly higher by 21.00%, but significantly lower by 13.33% and 25.48% at the jointing and V12 stages, respectively, as compared to the U treatment. Soil available potassium in the U+FA treatment was 75.41% higher than in the U treatment at the silking stage.

3.3. Endogenous Hormones and N Metabolism-Related Enzyme Concentrations in Maize Root

At the V12 stage of maize, IAA and ZR concentrations in maize root were significantly increased by 42.32% and 50.11%, respectively, in the U+FA treatment compared with the U treatment (Figure 1A). Meanwhile, there is a significant effect on NR, NiR, GS, GSR, GOGAT, and GLDH in U+FA treatment at the V12 stage. The concentrations of NR, NiR, GS, GOGAT, and GLDH in the maize root of the U+FA treatment were significantly higher by 74.17%, 16.61%, 45.60%, 43.07%, and 69.02%, respectively, compared with the U treatment (Figure 1B–D). The GSR concentration in the U+FA treatment was higher, though not significantly, than in the U treatment (Figure 1C). In summary, the concentrations of endogenous hormone and the N metabolism-related enzymes in the maize root of urea in combination with FA solution treatment were boosted compared to the U treatment.

Table 3. Effects of different fertilization treatments on soil nutrients in 2017. one-way ANOVAs followed by SNK's test at $p < 0.05$.

Variable (mg kg ⁻¹)	Treatment	Maize Growth Stages			
		Seedling	Jointing	12-Leaf	Silking
NH ₄ ⁺ -N	CK	13.78 a	8.92 b	3.31 ab	4.11 b
	U	18.56 a	8.09 b	3.13 b	3.38 b
	U+FA	15.58 a	19.37 a	3.51 a	16.65 a
NO ₃ ⁻ -N	CK	26.32 b	12.89 c	12.97 b	7.81 ab
	U	126.48 a	22.65 a	13.83 b	6.89 b
	U+FA	132.03 a	18.79 b	15.52 a	8.09 a
Available P	CK	54.65 c	83.31 a	108.23 a	31.50 b
	U	63.87 b	75.85 b	81.40 b	42.93 a
	U+FA	76.96 a	65.74 c	60.66 c	42.49 a
Available K	CK	343.29 a	116.46 b	244.38 a	144.41 b
	U	319.98 a	189.41 a	159.23 b	134.29 b
	U+FA	345.42 a	169.48 a	149.02 b	235.57 a

Treatments: no nitrogen fertilizer (CK); application of urea (U); combined application of urea and fulvic acid (U+FA). Values within a same column followed by different letters are significantly different between treatments for a same year according to least significant difference test ($p < 0.05$).

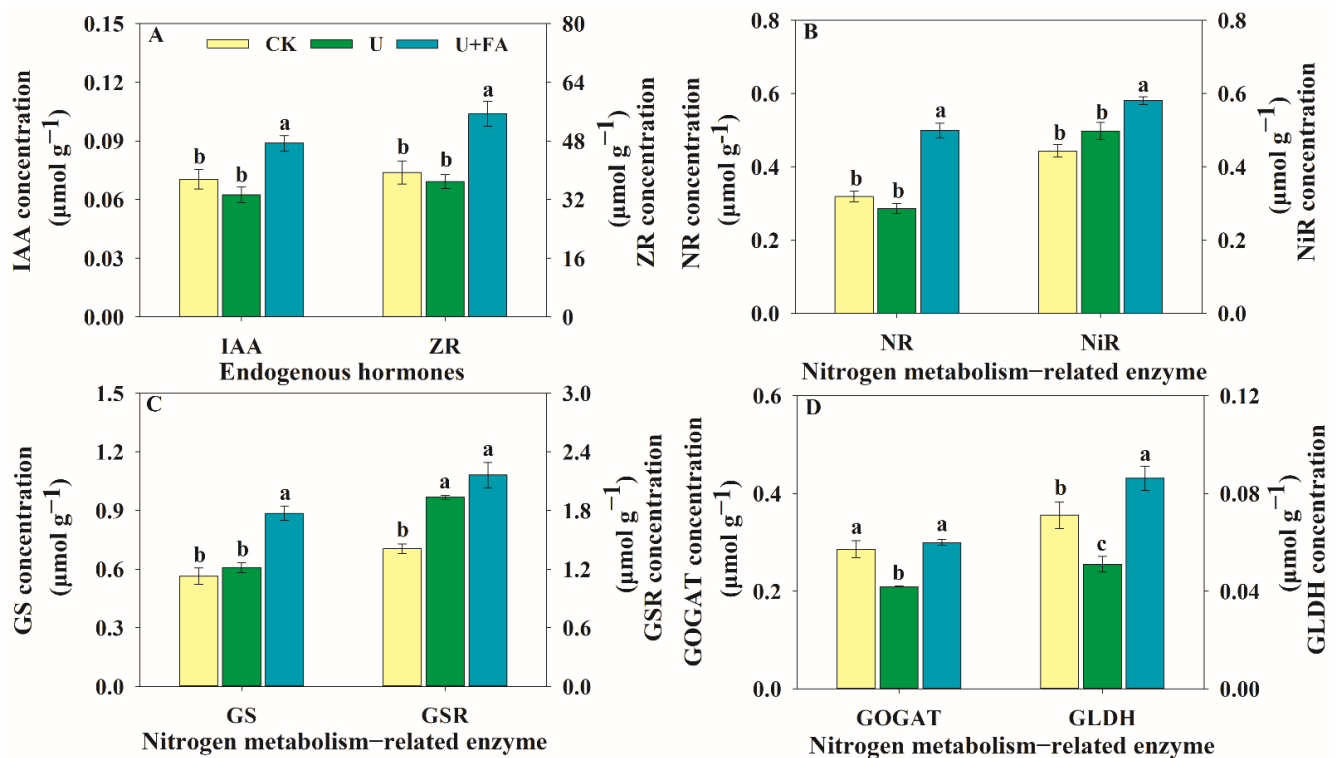


Figure 1. Effects of different fertilization treatments on the endogenous hormones of auxin (IAA, (A)) and zeatin nucleoside (ZR, (A)) and the concentrations of nitrogen metabolism-related enzymes of nitrate reductase (NR, (B)), nitrite reductase (NiR, (B)), glutamine synthetase (GS, (C)), glutathione reductase (GSR, (C)), glutamate synthase (GOGAT, (D)), and glutamate dehydrogenase (GLDH, (D)) in maize root at the 12-leaf stage, in 2018. The treatments include: no nitrogen fertilizer (CK); application of urea (U); combined application of urea and fulvic acid solution (U+FA). The values are means of four replicates, and the error bars are standard errors. Different letters indicate significant differences between treatments for a same parameter according to SNK's test at $p < 0.05$.

3.4. Growth Indexes and Photosynthesis of Maize Leave

Maize plant height increased with time during the growth season (Table 4). The plant height of the U+FA treatment was significantly advanced by 8.47% and 8.19%, at the V12

and silking stage, respectively, compared to the U treatment. Maize stem diameter first increased and then decreased during the growth season. At the jointing stage, maize stem diameter was 17.58% greater than in the U+FA treatment than in the U treatment. At the seedling, V12, and silking growth stages, there were no great disparities in stem diameter between the U and U+FA treatment.

Table 4. Effects of different fertilization treatments on plant height and stem diameter at the different growth stages of maize, in 2017.

Variable	Treatment	Maize Growth Stages			
		Seedling	Jointing	12-Leaf	Silking
plant height (cm)	CK	51.60 b	93.23 b	139.80 c	189.90 c
	U	60.86 a	109.33 a	227.08 b	231.45 b
	U+FA	60.28 a	111.15 a	246.33 a	250.40 a
stem diameter (mm)	CK	10.47 b	22.02 b	20.56 b	20.49 b
	U	13.06 a	23.31 b	26.50 a	24.68 a
	U+FA	13.47 a	27.41 a	26.77 a	25.06 a

Treatments: no nitrogen fertilizer (CK); application of urea (U); combined application of urea and fulvic acid (U+FA). Values within a same column followed by different letters are significantly different between treatments for a same year according to least significant difference test ($p < 0.05$).

At the jointing stage, the SPAD value and Pn of the U+FA treatment were significantly higher by 18.63% and 12.69%, respectively, and Gs was significantly lower by 19.63%, compared to the U treatment (Figure 2). There was no notable change in Tr between the U and U+FA treatments at the jointing stage (Figure 2B). The results of SPAD value, Pn, Gs, and Tr indicated improved CO₂ assimilation and photosynthate accumulation in the U+FA treatment as compared with the U treatment.

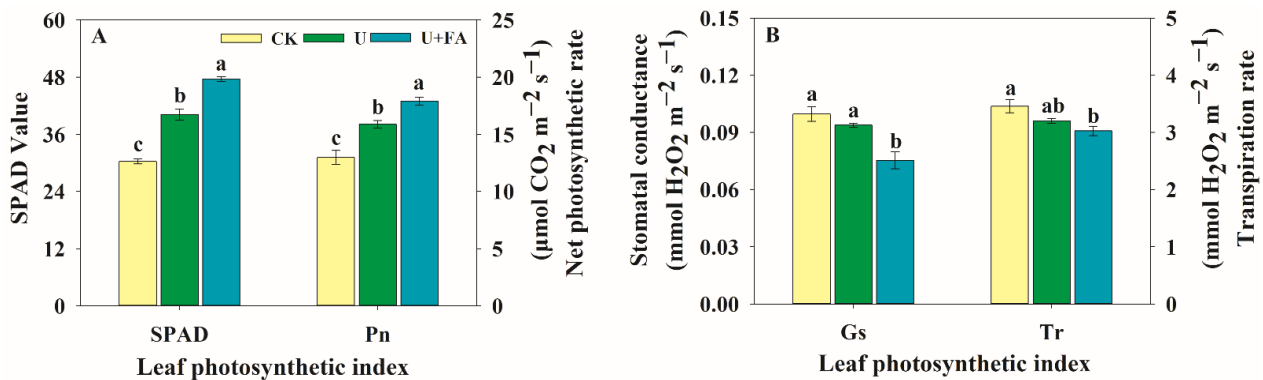


Figure 2. Effects of different fertilization treatments on SPAD value (A), net photosynthetic rate (Pn, (A)), stomatal conductance (Gs, (B)), and transpiration rate (Tr, (B)) in maize leave at the jointing stage, in 2018. The treatments include: no nitrogen fertilizer (CK); application of urea (U); combined application of urea and fulvic acid solution (U+FA). The values are means of four replicates, and the error bars are standard errors. Different letters indicate significant differences between treatments for a same parameter according to SNK's test at $p < 0.05$.

3.5. Concentrations of Endogenous Hormone and Photosynthetic Key Enzyme in Maize Leave

Plant endogenous hormones are essential for plant growth and development. At the jointing stage, the concentrations of endogenous hormones in maize leave were significantly different between the treatments (Figure 3). The IAA, GA, and ZR concentrations were significantly higher by 68.86%, 50.58%, and 55.22%, respectively, whereas the ABA concentration was significantly lower by 32.78% in the U+FA treatment than in the U treatment.

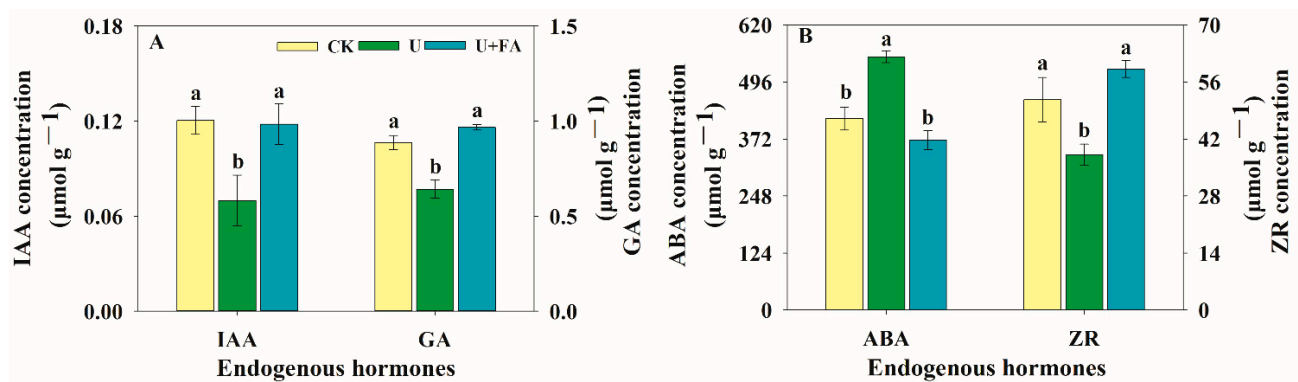


Figure 3. Effects of different fertilization treatments on the concentrations of auxin (IAA, (A)), gibberellin (GA, (A)), abscisic acid (ABA, (B)) and zeatin nucleoside (ZR, (B)) in maize leaf at the jointing stage, in 2018. The treatments include: no nitrogen fertilizer (CK); application of urea (U); combined application of urea and fulvic acid solution (U+FA). The values are means of four replicates, and the error bars are standard errors. Different letters indicate significant differences between treatments for a same parameter according to SNK's test at $p < 0.05$.

The PEPC and PPDK are two key enzymes in carbon assimilation. Their activities were significantly different between the treatments during the jointing and V12 stages (Figure 4). At the jointing stage, PEPC and PPDK activities were not significantly distinct between the U+FA and U treatments. However, at the V12 stage, the concentrations of PEPC and PPDK mostly in the U+FA treatment were significantly higher by 42.96% and 29.40%, respectively.

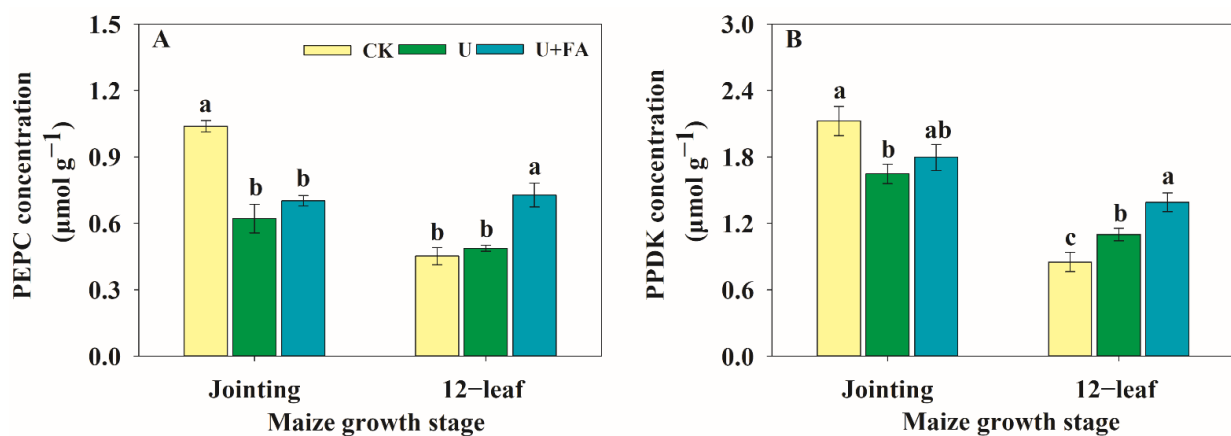


Figure 4. Effects of different fertilization treatments on the concentrations of photosynthetic key enzymes of phosphoenolpyruvate carboxylase (PEPC, (A)) and pyruvate phosphate dikinase (PPDK, (B)) in maize leaf at the jointing and V12 stages, in 2018. The treatments include: no nitrogen fertilizer (CK); application of urea (U); combined application of urea and fulvic acid solution (U+FA). The values are means of four replicates, and the error bars are standard errors. Different letters indicate significant differences between treatments for a same growth stage according to SNK's test at $p < 0.05$.

3.6. N Metabolism-Related Enzyme Concentrations in Maize Leaf

Nitrogen metabolism is an important physiological process in plant growth and development. The N metabolism in maize leaf was significantly influenced by the combined application of urea and FA solution (Figure 5). The GLDH concentration of the U+FA treatment was significantly higher than of the U treatment by 63.96% at the jointing stage (Figure 5A). At the V12 stage, the NR and GSR concentrations in the U+FA treatment were significantly enhanced by 73.16% and 65.13%, respectively, as compared to

the U treatment (Figure 5B,C). The NiR concentration was significantly increased by 38.38% and 63.93% at the jointing and V12 stages, respectively, in the U+FA treatment compared to the U treatment (Figure 5D). The GS/GOGAT cycle is a prominent pathway of ammonium assimilation in plants [41]. By comparing the U+FA treatment to the U treatment at the jointing and V12 stages, GS concentration was significantly higher by 27.86% and 50.63%, respectively, and GOGAT concentration was significantly higher by 31.83% and 43.07%, respectively (Figure 5E,F).

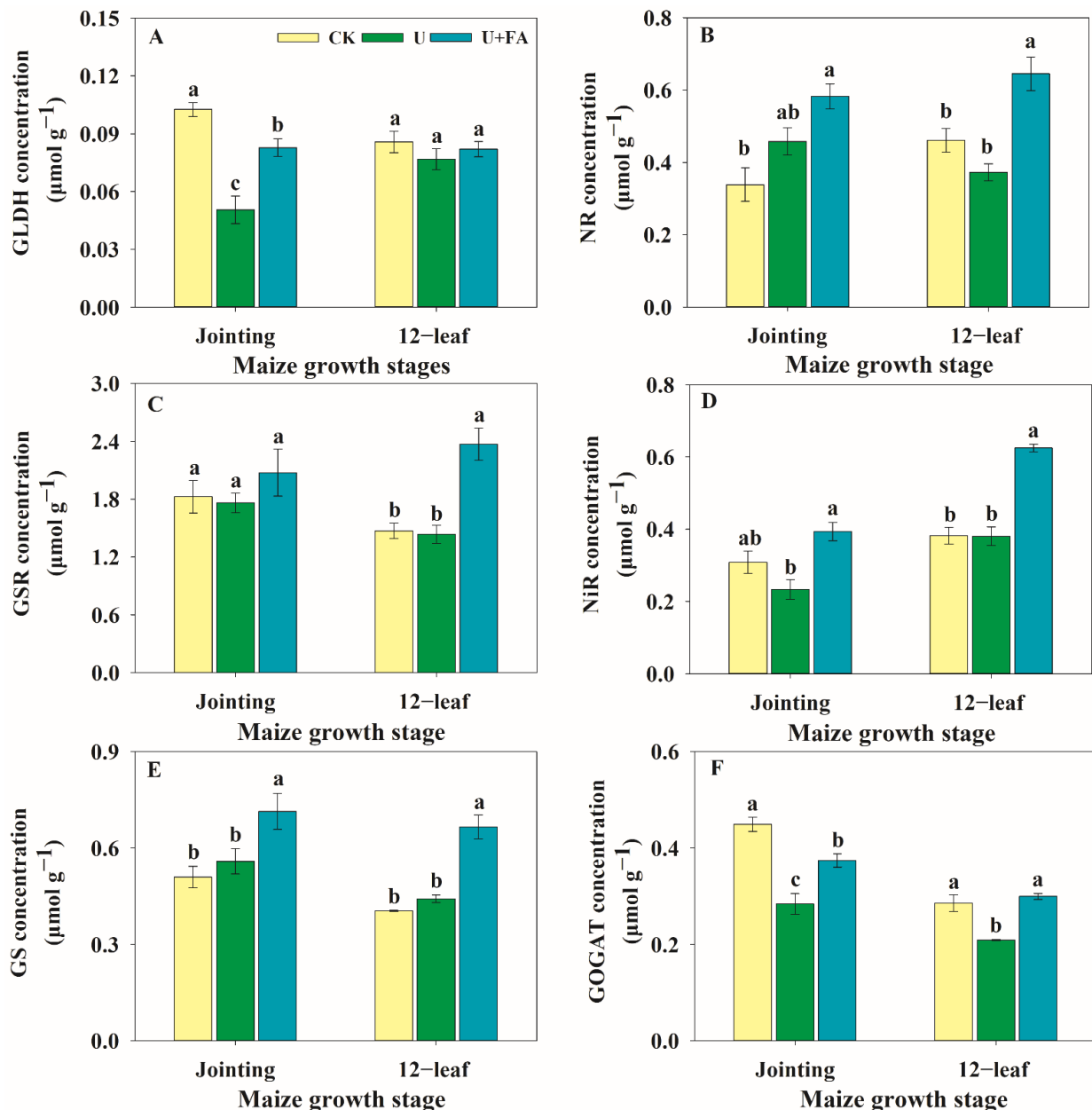


Figure 5. Effects of different fertilization treatments on the concentrations of N metabolism-related enzymes of glutamate dehydrogenase (GLDH, (A)), nitrate reductase (NR, (B)), glutathione reductase (GSR, (C)), nitrite reductase (NiR, (D)), glutamine synthetase (GS, (E)), and glutamate synthase (GOGAT, (F)) in maize leaves at the jointing and V12 stages, in 2018. The treatments include: no nitrogen fertilizer (CK); application of urea (U); combined application of urea and fulvic acid solution (U+FA). The values are means of four replicates, and the error bars are standard errors. Different letters indicate significant differences between treatments for a same growth stage according to SNK's test at $p < 0.05$.

3.7. Multi-Dimensional Correlation Analysis

It shows the correlations between maize yield and soil inorganic nitrogen contents, Gs, endogenous hormone concentrations, nitrogen metabolism-related enzyme activities, and photosynthetic enzyme activity (Figure 6A). There were significant correlations between maize yield and soil NH_4^+ -N and NO_3^- -N contents. The combined application of urea and FA in the U+FA treatment provided sufficient nitrogen to meet the demand of maize during its critical growth stages, resulting in significantly higher yield. Plant endogenous hormones greatly influence plant growth and development, and they are linked to carbohydrate accumulation and yield. At the V12 stage, the IAA concentrations of maize root and leaves were significantly larger in the U+FA treatment than in the U treatment, stimulating the growth of root and the elongation of stems. Furthermore, maize yield was significantly related to Gs, GOGAT, IAA, NR, and GS in leaves and NR in root. The higher concentrations of nitrogen metabolism-related enzymes (e.g., GOGAT, NR, and GS) in the U+FA treatment indicate more active nitrogen assimilation and metabolism.

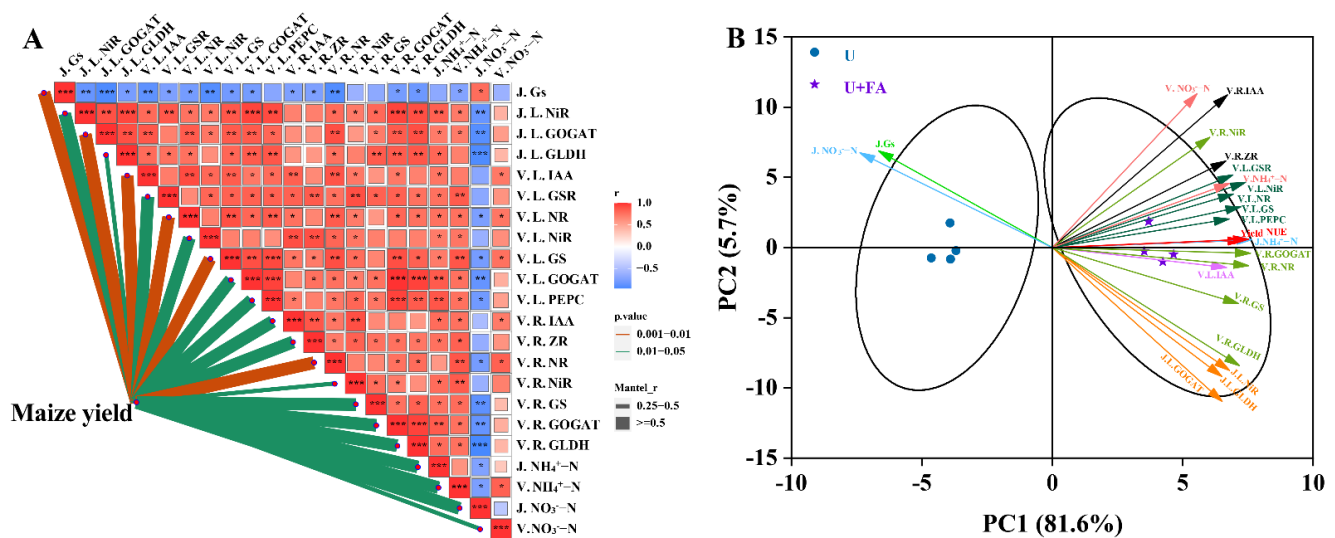


Figure 6. The correlations between maize yield, concentrations of nitrogen metabolism-related enzymes, concentrations of photosynthetic key enzymes, concentrations of endogenous hormones, and soil available nutrients. (A) Mantel test. The thickness of a line indicates the strength of the correlation. The area of a rectangle represents the magnitude of the correlation. Red denotes a positive correlation and blue represents a negative correlation. “*”, “**” and “***” indicate significant correlation at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. (B) Principal component analysis showing the relationship among yield, NUE, soil nutrients, concentrations of nitrogen metabolism-related enzymes, concentrations of photosynthetic key enzymes of different treatment. (J): jointing stage; V: 12-leaf stage; L: leaves of maize; R: root of maize; Gs: stomatal conductance; IAA: auxin; ZR: zeatin nucleoside; NR: nitrate reductase; NiR: nitrite reductase; GS: glutamine synthetase; GSR: glutathione reductase; GOGAT: glutamate synthase; GLDH: glutamate dehydrogenase; Pn: net photosynthetic rate; Gs: stomatal conductance; Tr: transpiration rate; GA: gibberellin; ABA: abscisic acid; PEPC: phosphoenolpyruvate carboxylase).

As demonstrated by the correlation analysis, the results of the PCA confirmed the Mantel test (Figure 6B). The PCA indicated that the 23 variables were divided into PC1 (81.6%) and PC2 (5.7%). The contribution of FA to responding to these parameters was clear when compared to treatments of U. Meanwhile, the concentration and activities of C and N metabolism-related enzymes were significantly affected by U combined with FA solution. In addition, NO_3^- -N content and Gs were dispersed in the second quadrant and had a negative relationship with the other parameters, which were mostly distributed in the first and fourth quadrants. There was a strong correlation between maize yield and PEPC. The PEPC concentration in leaves was significantly greater in the U+FA treatment.

It's possible that U+FA treatment increased the activities of N metabolism-related enzymes, which increased the uptake of maize root nutrients and then affected the concentration of key enzymes in shoot photosynthesis, promoting the TCA cycle, increasing dry matter accumulation, and eventually increasing yield.

4. Discussion

4.1. Effects of Urea–FA Solution Combined Application on Soil Available Nutrients, Maize Yield, and NUE

Organic wastes include crop straws and livestock manure, which can be used as raw materials for aerobic degradation to produce biochemical humic substances. The structure of humic substance shifted over time, including a wide range functional groups—such as carboxylic, phenolic and hydroxylic groups, during anaerobic process [42,43]. Fulvic acid (FA) is one of the dominant components of humus [44]. Originated from plant residues, FA is a type of organic polymer, containing carboxyl, quinone, phenolic hydroxyl, amide, and other functional groups, in this study (Figure S1). The phenolic hydroxyl and quinone groups in FA react with the sulfhydryl groups in urease, urease activity would be inhibited and ammonia volatilization from urea would be reduced [45]. In addition, the carboxyl and phenolic hydroxyl groups in FA interact with the amide groups in urea through coordination complexation, ion exchange, or hydrogen bond to form stable FA-urea complexes, delaying the decomposition of urea. The phenol hydroxyl and carboxyl groups in FA react with NH_4^+ to generate stable FA-ammonium salts [46–48], improving the availability of NH_4^+ to meet plant nitrogen demand at the critical growth stages. In this study, soil NH_4^+ was significantly increased (Table 3), and maize grain yield and NUE were significantly improved by 12.61% and 20.70 percentage points, respectively, in the U+FA treatment compared with the U treatment (Table 2).

Due to its loose, complex “sponge-like” structure and rich functional groups [49,50], FA has a large specific surface area and a high adsorption capacity. Its carboxyl and phenolic hydroxyl groups can adsorb potassium ions to reduce potassium fixation by clay minerals [51,52]. Compared to the U treatment, soil available potassium of the U+FA treatment was significantly higher (Table 3), contributing to the better maize growth in the treatment. Furthermore, the presence of FA alters the distribution of root exudate profile and it also tend to increase the release of oxalate and citrate from maize root [53–55]. Additionally, H₂ is produced during FA decomposition [56], resulting in an available P increase in calcareous soils and the dissolution of insoluble P compounds. On the other hand, the adsorption of FA on the adsorption plane creates a repulsive negative electric potential and a steric hindrance on the mineral surface, which further inhibit P binding on the soil surface [57]. Furthermore, the carboxyl, hydroxyl, and alcoholic hydroxyl groups in FA can complex with such metal ions as Ca^{2+} and Mg^{2+} , decreasing the fixation of phosphorus in soil and thereby increasing its mobility and availability [58–60]. Compared with the U treatment, the soil available phosphorus in the U+FA treatment was significantly higher at the seedling stage (Table 3), which would improve the vegetative growth and contribute to the later reproductive growth of maize in this treatment. In a word, the combined application of FA with urea in the U+FA treatment resulted in higher soil nutrient availability, which stimulated maize growth and in turn increased yield.

4.2. Effects of Urea–FA Solution Combined Application on the Endogenous Hormones and N Metabolism-Related Enzyme Concentrations in Maize

Plant endogenous hormones are important growth-regulating factors that respond to the changing environment. The hormone IAA triggers root cell signals, up-regulates H^+ -ATPase gene expression, and activates the enzymes and proteins related to cell wall relaxation and extension [61]. Compared with the U treatment, the U+FA treatment displayed significantly higher IAA concentrations in maize root at the V12 stage (Figure 1), indicating more active root development in this treatment. In addition, IAA often works synergistically with GA to influence cell expansion and tissue differentiation [62,63]. Compared with the U treatment, the U+FA treatment displayed higher levels of IAA and GA in maize

leave at the jointing stage (Figure 3), indicating better growth and development of the leaves in this treatment. Therefore, the combining urea and FA solution resulted in higher concentrations of endogenous hormones, which stimulated the growth and development of maize.

Aliphatic carboxylic acids and aromatic carboxylic acids are two structural components of FA. Similar to plant hormones [64,65], they can induce the expression of N transporter genes (ZmNrt2.1 and ZmNrt1.1) and H^+ -ATPase subtypes (Mha1 and Mha2) [66]. Therefore, they can regulate N metabolism, boost the concentrations of NR, NiR, GS, and GOGAT, and enhance the nutrient absorption capacity of maize [67,68]. The enzymes NR and NiR catalyze the transformation of NO_3^- to NO_2^- and NH_4^+ and regulate the absorption and utilization of nitrogen by plants. The enzymes GS and GOGAT catalyze the reaction between glutamine and α -ketoglutarate to produce glutamate [11], which can be transported into the root tip meristem through extracellular transport, promoting root cell division and nutrient absorption [10]. Compared with the U treatment, the U+FA treatment displayed significantly enhanced concentrations of NR, NiR, GS, and GOGAT in maize root at the V12 stage. Furthermore, Mantel test revealed that the activities of N metabolism-related enzymes were significantly correlated with plant endogenous hormones and yield. In summary, the U+FA treatment increased the concentrations of N metabolism-related enzymes, which stimulated N metabolism, promoted plant growth, and contributed to yield increase.

4.3. Effects of Urea–FA Solution Combined Application on Photosynthetic Enzyme Concentrations

Maize is a C_4 plant with high photosynthetic efficiency, with 80% of the grain dry matter being leaf photosynthate. The PEPC and PPDK in maize leave are the key enzymes involved in photosynthetic carbon reduction [69–71]. In mesophyll cells, PEPC immobilizes CO_2 into oxaloacetic acid [72], providing a carbon skeleton for carbohydrate biosynthesis and nitrogen assimilation. The enzyme PPDK phosphorylates pyruvate into phosphoenolpyruvate, which is the primary CO_2 acceptor, promoting the tricarboxylic acid cycle. Studies showed that FA directly affects CO_2 assimilation and promotes carbohydrate accumulation by increasing the mRNA transcription and translation of PEPC and PPDK in cells [73,74]. In this study, the concentrations of PEPC and PPDK in the U+FA treatment were significantly higher than in the U treatment, explaining the higher aboveground dry biomass and grain yield in the former.

Significantly positively correlated with chlorophyll concentration, SPAD values can be used to estimate chlorophyll concentrations [1]. According to Alla and Hassan [75], FA can up-regulate the ALAD gene (AFW69390.1) and induce the expression of H^+ -ATPase subtypes (Mha1 and Mha2) in maize, significantly improving the photoreaction of photosynthesis and expediting carbon cycling. In this study, the U+FA treatment significantly increased the SPAD value and photosynthetic rate at the jointing stage compared with the U treatment. In summary, the activities of carbon cycle-related enzymes and SPAD value were significantly increased in the U+FA treatment, which explains the higher aboveground dry biomass and grain yield in this treatment.

As we all know, applying fertilizer can improve crop growth, and applying urea combined with FA solution can partially improve crop growth because the treatment is more efficient. Through this experiment, we found that the nutrient availability was increased by combining urea with FA solution and could meet the absorption of nutrients by plants. At the same time, by measuring various physiological and biochemical indicators, we found that the content of endogenous hormones and nitrogen cycle-related enzymes in urea combined with FA solution was significantly higher than that in urea. At present, we have come to the conclusion that the combined application of urea and FA solution significantly improved soil nutrient availability, increased the concentrations of endogenous hormones in maize, stimulated the activities of enzymes related to nitrogen metabolism, promoted the photosynthetic carbon assimilation efficiency, and ultimately improved the crop yield and NUE. At the same time, the mechanism of the significant expression of genes related to the nitrogen cycle in urea and FA solution will be continued in the future.

5. Conclusions

The combined application of urea and fulvic acid solution improved soil ammonium nitrogen, nitrate nitrogen, available phosphorus, and available potassium, increased the concentrations of auxin and zeatin nucleoside in maize leaf and root, decreased the level of abscisic acid in maize leaf, and stimulated the activities of nitrogen metabolism and photosynthesis enzymes, resulting in higher above-ground dry biomass, maize yield, and nitrogen use efficiency.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12061400/s1>, Figure S1. Fourier transform infrared spectrum of FA. There is a peak at 3440 cm^{-1} , which is attributed to -COOH . The peaks at 3348 and 1153 cm^{-1} are due to -OH . The peak at 1680 cm^{-1} is attributed to -C=O- . The other two peaks at $1620\text{--}1679\text{ cm}^{-1}$ are due to -C=O- as well. The peak at $1153\text{--}1465\text{ cm}^{-1}$ is attributed to R-O-R . All authors have read and agreed to the published version of the manuscript.

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