Article

Grain Yield, Nitrogen Use Efficiency and Antioxidant Enzymes of Rice under Different Fertilizer N Inputs and Planting Density

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Abstract: Nitrogen fertilizer and planting density are key factors affecting rice yield and nitrogen utilization efficiency. There is still a need to optimize nitrogen fertilizer and planting density management for high yield. We studied the effects of different quantities of nitrogen application (N_0 0 kg ha⁻¹, N_1 120 kg ha⁻¹, N_2 180 kg ha⁻¹) and planting density (low-density: 18.8 hills m⁻²; high-density: 37.5 hills m⁻²) on rice yield, photosynthetic characteristics, antioxidant system, and nitrogen use efficiency. ANOVA results indicated that most tested traits were affected by environment, planting density, N application, and their interactions. Comparing the results of low-density planting, high-density planting increased the panicle number by 21.12% but decreased the grain number per panicle and yield by 3.97% and 22.48%, respectively. Similarly, the superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) activities and PFPn (partial factor productivity of nitrogen) decreased by 8.20%, 6.99%, 16.41%, 14.28%, and 5.73%, respectively, while HIn (N harvest index) increased by 1.32%. Compared to no N application, N application treatments increased the panicle number, grain number per panicle, and yield by 9.92%, 17.64%, and 37.83% in the N_1 treatment; and increased by 17.15%, 29.09%, and 128.94% in the N_2 treatment, respectively. N application significantly increased net photosynthetic rates and enzyme activities of antioxidant antioxidases. Compared with N_1 , N_2 decreased REn (apparent recovery efficiency of N), AEn (agronomic N use efficiency), and PFPn by 8.98%, 11.80%, and 25.14%, respectively, while, compared with N_0 , N_1 increased HIn by 8.50%. It was observed that nitrogen fertilizer and planting density had an interaction effect on the net photosynthetic rate, antioxidant enzyme activities, and PFPn and HIn. Given a comprehensive consideration, it is best to apply nitrogen at a rate of 120 kg ha⁻¹ at the planting density of 37.5 hills m⁻² for high grain yield and high N use efficiency in our experimental site.

Keywords: planting density; grain yield; nitrogen use efficiency; photosynthetic characteristics; antioxidant system

Citation: Wang, W.; Shen, C.; Xu, Q.; Zafar, S.; Du, B.; Xing, D. Grain Yield, Nitrogen Use Efficiency and Antioxidant Enzymes of Rice under Different Fertilizer N Inputs and Planting Density. *Agronomy* **2022**, *12*, 430. <https://doi.org/10.3390/agronomy12020430>

Received: 19 November 2021

Accepted: 21 January 2022

Published: 9 February 2022

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1. Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops globally and the staple food of nearly half of the world's population, so it plays an important role in global food production and security [1]. An increase in rice yield will play an important role in future food production. The sustainable and healthy development of rice production and the steady growth of rice yield contribute to national food security [2]. With the acceleration

of urbanization, the arable land area is decreasing, and the increase in rice yield must be obtained by improving productivity by increasing the yield per unit area [3,4].

Nitrogen (N) is critical to the growth and development of rice and participates in many metabolic processes, such as protein hydrolysis and amino acids metabolism [3]. Nitrogen uptake is affected by various characteristics, fertilizer application, soil conditions, and environmental factors [5]. To meet the increasing demand for food in China, a large amount of nitrogen fertilizer is applied during agricultural production to obtain a greater yield [6]. However, excessive nitrogen fertilizer application has increased surface runoff and nitrification, resulting in N loss [7,8]. At present, the average recovery efficiency of nitrogen fertilizer is 33% at the field level [9]. Losses of N from the rice system would hamper the N use efficiency and could cause a series of environmental problems, including water eutrophication, soil hardening, acid rain, and the greenhouse effect [10,11]. In addition, it increases the sensitivity of crops to lodging and disease, and it limits the opportunities to increase rice yields [12]. Therefore, it is vital to improve crop N use efficiency to achieve a high yield [13].

The transplanting density of rice is the initial growth and development of a rice population, which can significantly affect the yield, population growth, and development of rice [14]. It is reported that increasing the transplanting density can increase the effective panicle number and yield of rice [15], and reducing transplanting density can increase the number of grains per spike and yield [16]. Huang et al. [17] found that increased density could compensate for yield loss caused by reduced N inputs. High transplanting density could increase the accumulation of dry matter in the early stage of growth and development [18], which might be attributed to the greater potential of crop canopies to capture solar radiation, water, and nutrients in the deeper soil layer [19]. Nevertheless, an excessive transplanting density of rice will reduce the distances between rows and between plants, and increase the length of the internode at the base of the stem, which will reduce the flexural strength of the stem and increase the risk of rice lodging [20]. An appropriate reduction of rice transplanting density can significantly improve the physical and chemical properties of rice stems and enhance their lodging resistance. However, it has little effect on the material accumulation in the middle and late stages, and even caused the reduction of dry matter accumulation in the middle and late stages of rice growth and development [21]. A slight increase of rice row spacing can significantly improve the canopy permeability, light interception ability, and light energy utilization efficiency of rice, leading to an increase in photosynthetic capacity and material accumulation capacity of rice leaves in the middle and later stages of growth and development [22]. As for the suitable transplanting density of rice, research results have been inconsistent to date. Generally speaking, planting rice close together will be more conducive to developing rice yield potential.

Appropriate rice transplanting density and a reasonable combination of nitrogen fertilizer application levels can increase the nitrogen fertilizer absorption and utilization of rice, affecting the growth efficiency and yield with an interacting effect [23,24]. From the perspective of protecting the environment, several researchers have increased the transplanting density and reduced the amount of nitrogen application, which improved the utilization rate of nitrogen fertilizer, and reduced the pollution of nitrogen fertilizer to the environment [17,24–28]. However, to achieve higher yields and lower pollution levels, there is still room for optimization in selecting planting density and nitrogen application rates. Previous studies have focused on yield increase and nitrogen use efficiency, while the effects of planting density and nitrogen application rate on rice physiology were rarely reported. Here, we investigated the effects of planting density and the nitrogen application rate on rice photosynthesis, nitrogen use efficiency, and yield in Jingzhou city, Hubei Province, China, from 2017 to 2019.

2. Material and Methods

2.1. Experimental Site Description

We conducted experiments from 2017 to 2019 at the Yangtze University Farm, Jingzhou County (30.33° N, 112.23° E, 36 m), Hubei Province, China. The area lies in the northern subtropical agricultural climate zone. The annual average temperature is 16.5 °C, the accumulated temperature ≥ 10 °C is 5094.9–5204.3 °C, the annual average precipitation is 1095 mm, and the sunshine rate is 1718 h. Meteorological data from 2017 to 2019 are shown in Figure 1. The soil type was sandy loam, and the soil characteristics are shown in Table 1. Chang-nong-geng 1 (CNG1) bred by Yangtze University was used as material in this study, which possesses excellent rice quality and high yield with a growth duration of 124–130 days from sowing to maturity.

Figure 1. Meteorological data from 2017–2019. $T_{\max}$, $T_{\min}$, T_{mean} , and Pre are the maximum temperature, minimum temperature, average temperature, and precipitation, respectively.

Table 1. Average soil characteristics ($n = 3$) of experiment field composite topsoil samples (0–20 cm). The experiment was conducted at Yangtze University farm, Jingzhou County, Hubei Province, China (2017–2019).

Year	Total N (g kg ^{−1})	Available N (mg kg ^{−1})	Total P (g kg ^{−1})	Total K (g kg ^{−1})	Organic Matter (g kg ^{−1})	pH
2017	2.24	83.94	0.57	3.49	21.61	6.80
2018	2.27	87.16	0.53	3.55	21.41	6.70
2019	2.25	86.88	0.59	3.67	21.39	6.70

2.2. Experimental Treatment and Design

The experiment was conducted from 2017 to 2019 and laid out in a split-plot design with planting density as the main plot and nitrogen application as the subplot. Two planting

densities and three nitrogen application rates were tested with three replicates, totaling 18 subplots ($4.5\text{ m} \times 6.5\text{ m}$) in the field. The planting densities were 37.5 and 18.8 hills m^{-2} , representing hill spacings of $13.3 \times 20\text{ cm}$ and $26.7 \times 20\text{ cm}$, respectively. The nitrogen application rates were 0, 120, and 180 kg N ha^{-1} . Nitrogen in the form of urea was split-applied with 40% as a basal fertilizer, 30% at mid-tillering, and 30% at flowering stages. P and K fertilizers were applied as a basal fertilizer (kg ha^{-1}): P_2O_5 59 and K_2O 120 were applied in the form of $(\text{NH}_4)_2\text{HPO}_4$ and KCl, respectively.

Rice was sown on May 1 and transplanted on June 1 in 2017–2019. One or two seedlings were transplanted per hill in each experiment. Each main plot was surrounded by a 35 cm wide ridge covered with a plastic film. The plastic film was installed to a depth of 20 cm below the soil surface three days before transplanting. Weeds, pests, and diseases were intensively controlled to avoid yield loss.

2.3. Measurement Items and Methods

2.3.1. Yield and Yield Components

Grain yields were measured at maturity by taking 5 m^2 plant samples at the center of each plot. Grains were separated from the rachis, filled and unfilled grains were separated, and the total weight of filled grains was determined. Filled grains were dried in an oven at 70 °C to a constant weight, and grain yield was calculated at 14% moisture content. Plant samples were taken to determine the yield components (panicle density, number of spikelets per panicle, 1000-grain weight, and seed setting rate) from 12 hills (0.48 m^2) adjacent to the harvest area.

2.3.2. Photosynthetic Rate

The photosynthetic rate of the top fully-expanded leaves of the mainstem was determined at the mid-tillering, heading, and maturity stages. Five leaves were chosen for each treatment. A gas exchange analyzer (Li-6400, Li-COR Inc., Lincoln, NE, USA) was used to take the measurements between 9:30–11:00 a.m., when the active photosynthetic radiation above the canopy was 1200 $\text{mmol m}^{-2}\text{ s}^{-1}$.

2.3.3. Antioxidase

The fresh flag leaf samples at the panicle initiation stage were ground with liquid nitrogen to form homogenate, and 9 mL of 50 mM sodium phosphate buffer (pH 7.8) was added to the homogenate and centrifuged at 8000 rpm for 15 min at 4 °C. The activities of superoxide dismutase (SOD) at 560 nm, catalase (CAT) at 240 nm, peroxidase (POD) at 470 nm, and ascorbate peroxidase (APX) at 290 nm were determined according to the methods of Du et al. [29].

2.3.4. Nitrogen Use Efficiency

The grain and straw harvested at maturity were used for tissue N analysis after drying at 80 °C to a constant weight and weighed. N content in the grains and straw was determined with an automatic Kjeldahl Apparatus (FOSS-8400, Höganäs, Sweden). The apparent recovery efficiency of N (RE_N), agronomic N use efficiency (AE_N), partial factor productivity of applied N (PFP_N), physiological N use efficiency (PE_N), internal N use efficiency (IE_N), and N harvest index (HI_N) were calculated according to the method of Gu et al. [30].

2.4. Statistical Analyses

Statistical data were analyzed using variance tests (ANOVA) in SPSS 21.0, and the differences between treatments were separated using the least significant difference (LSD) tests at 5% probability. The figure was drawn by Origin 9.1 (OriginLab Inc., Northampton, MA, USA).

3. Results

3.1. Effect of Nitrogen Application and Planting Density on Yield and Yield Components

Table 2 shows summary statistics of the grain yield and its components under two density and three N applications across years. ANOVA results indicated that the differences among years and N applications, between densities, and variances due to interactions between any two factors (year, density, and N application) and among the three factors were significant or highly significant for most tested traits. Density had significant effects on the number of panicles, the number of grains per panicle, thousand-grain weight, and rice yield. Under different nitrogen treatments, the trend of panicle number and yield was LD < HD, though other traits show HD < LD. Compared with low-density planting, high-density planting increased the panicle number and yield by 21.12% and 22.48% and decreased the grain number per panicle by 3.97%, respectively. Nitrogen application had a significant impact on the number of ears, grains per ear, thousand-grain weight, and rice yield. Under different density treatments with the trend of $N_0 < N_1 < N_2$. Nitrogen had a significant impact on the seed setting rate with a trend of $N_2 < N_1 < N_0$. Compared with no N application, the N1 treatment increased the panicle number, grain number per panicle, and yield by 9.92%, 17.64%, and 37.83%, respectively, while the N2 treatment increased the panicle number, grain number per panicle, and yield by 17.15%, 29.09%, and 128.94%, respectively. These results indicated that yield decreased with planting density while increased with N application.

Table 2. Effect of nitrogen application and planting density on yield and yield components. Different lowercase letters after the same column of values represented significant differences ($p < 0.05$) in the same year according to LSD. Values are the mean $\pm$ standard error ($n = 3$). ns: denotes non-significance; *, ** and ***: denote significance at $p < 0.05$, 0.01 and 0.001, respectively.

Year	Density	Nitrogen	Panicle Number (m ²)	Grain Number per Panicle	Seed Setting Rate (%)	1000-Grain Weight (g)	Grain Yield Kg ha ⁻¹
2017	Low	0	243.02 $\pm$ 21.32 d	107.08 $\pm$ 6.07 c	86.85 $\pm$ 1.94 a	27.93 $\pm$ 1.12 ab	6875.18 $\pm$ 301.54 e
	Low	120	277.58 $\pm$ 10.59 cd	125.52 $\pm$ 7.33 ab	86.37 $\pm$ 1.31 a	28.30 $\pm$ 0.74 a	9399 $\pm$ 513.06 c
	Low	180	309.40 $\pm$ 38.32 bc	141.38 $\pm$ 3.47 a	82.13 $\pm$ 2.25 b	28.33 $\pm$ 0.74 a	12,020.45 $\pm$ 672.57 a
	High	0	312.99 $\pm$ 15.69 abc	102.46 $\pm$ 12.57 c	84.71 $\pm$ 2.80 ab	26.48 $\pm$ 0.87 b	8313.39 $\pm$ 338.08 d
	High	120	340.72 $\pm$ 11.14 ab	117.22 $\pm$ 9.62 bc	84.47 $\pm$ 1.76 ab	26.98 $\pm$ 1.11 ab	10,275.74 $\pm$ 467.44 b
	High	180	355.27 $\pm$ 20.42 a	129.74 $\pm$ 12.72 ab	84.05 $\pm$ 0.66 ab	27.79 $\pm$ 0.24 ab	12,142.64 $\pm$ 761.39 a
2018	Low	0	267.33 $\pm$ 23.45 d	103.46 $\pm$ 4.96 c	80.02 $\pm$ 3.19 a	26.30 $\pm$ 1.40 a	6760.89 $\pm$ 293.81 d
	Low	120	305.34 $\pm$ 11.65 c	123.27 $\pm$ 1.39 ab	76.46 $\pm$ 3.75 ab	27.60 $\pm$ 0.72 a	9907.6 $\pm$ 381.05 b
	Low	180	340.34 $\pm$ 23.67 b	133.89 $\pm$ 13.77 a	73.58 $\pm$ 3.13 b	27.97 $\pm$ 1.66 a	11,944.88 $\pm$ 776.25 a
	High	0	344.29 $\pm$ 17.26 b	106.34 $\pm$ 4.90 c	79.29 $\pm$ 3.05 ab	27.09 $\pm$ 0.37 a	9202.4 $\pm$ 562.99 c
	High	120	374.79 $\pm$ 12.26 ab	120.82 $\pm$ 3.88 b	77.39 $\pm$ 1.08 ab	27.46 $\pm$ 0.32 a	11,777.75 $\pm$ 525.56 a
	High	180	390.79 $\pm$ 22.46 a	125.31 $\pm$ 1.60 ab	75.57 $\pm$ 3.32 ab	26.71 $\pm$ 0.64 a	12,085.52 $\pm$ 573.21 a
2019	Low	0	239.94 $\pm$ 10.06 d	96.59 $\pm$ 7.13 d	78.67 $\pm$ 3.07 a	28.07 $\pm$ 0.99 a	5890.52 $\pm$ 346.88 f
	Low	120	271.25 $\pm$ 9.75 c	125.41 $\pm$ 6.55 ab	76.41 $\pm$ 1.78 ab	27.42 $\pm$ 0.92 ab	8335.34 $\pm$ 517.67 d
	Low	180	305.33 $\pm$ 24.31 b	133.15 $\pm$ 8.69 a	76.52 $\pm$ 1.31 ab	28.24 $\pm$ 0.36 a	10,792.49 $\pm$ 361.78 b
	High	0	322.86 $\pm$ 10.08 ab	100.18 $\pm$ 7.62 cd	76.46 $\pm$ 2.25 ab	26.05 $\pm$ 0.65 b	7772.91 $\pm$ 403.85 e
	High	120	332.45 $\pm$ 0.91 a	112.53 $\pm$ 5.54 bc	77.10 $\pm$ 1.71 ab	27.72 $\pm$ 0.51 ab	9581.52 $\pm$ 447.51 c
	High	180	326.05 $\pm$ 11.57 ab	131.88 $\pm$ 13.03 a	73.56 $\pm$ 2.89 b	28.06 $\pm$ 1.82 a	11,692.59 $\pm$ 420.98 a
ANOVA							
Year (Y)			***	**	***	*	***
Density (D)			***	*	ns	***	***
Nitrogen (N)			***	***	***	***	***
Y $\times$ D			*	ns	**	**	***
Y $\times$ N			ns	*	**	**	***
D $\times$ N			***	**	***	ns	***
Y $\times$ D $\times$ N			***	ns	***	***	*

3.2. Effect of Nitrogen Application and Planting Density on Net Photosynthetic Rate

Table 3 shows the summary statistics of the net photosynthetic rate at different growth stages under two density and three N applications over years. ANOVA results indicated that the differences among years and N applications, between densities, and variances due to interactions between any two factors (year, density, and N application) and among the three factors were significant or highly significant for most tested traits. With the maturity of rice, the net photosynthetic rate increased first and then decreased, showing $N_0 < N_2 < N_1$, and the performance was the same for three years (Table 3). At low density, the net photosynthesis of high-nitrogen treatment was higher than that of low-nitrogen treatment, showing $N_0 < N_1 < N_2$, and the trend was the same for three years. At high densities, net photosynthesis increased first and then decreased, consistent with the performance of rice reproductive stages in different years. Compared with no N application, N application increased net photosynthetic rates at the mid-tillering, heading, and maturity stages by 31.77%, 21.89%, and 15.49%, respectively. There was no significant difference in net photosynthetic rates between the N_1 and N_2 treatments. Under N_0 and N_1 treatments, overall, the net photosynthetic rate of low density was lower than that of high density, but the net photosynthesis rate under N_1 treatment did not increase with the increase of density. Under N_2 treatment, the net photosynthetic rate at low density was higher than that at high density. Compared with low-density planting, high-density planting led to decreased net photosynthetic rates at the mid-tillering and heading stages by 4.99% and 5.28%, respectively. These results showed that increased planting density reduced the net photosynthetic rate and that the net photosynthetic rate increased to some extent with N application.

Table 3. Effect of nitrogen application and planting density on net photosynthetic rate (Pn) ($\mu\text{M CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) at different growing stages. Different lowercase letters after the same column of values represented significant differences ($p < 0.05$) in the same year according to LSD. Values are the mean $\pm$ standard error ($n = 3$). ns: denotes non-significance; *, ** and ***: denote significance at $p < 0.05$, 0.01 and 0.001, respectively.

Year	Density	Nitrogen	At Mid-Tillering $\mu\text{M CO}_2 \text{ m}^{-2} \text{ s}^{-1}$	At Heading $\mu\text{M CO}_2 \text{ m}^{-2} \text{ s}^{-1}$	At Maturity $\mu\text{M CO}_2 \text{ m}^{-2} \text{ s}^{-1}$
2017	Low	0	18.80 $\pm$ 0.35 d	22.40 $\pm$ 0.17 cd	16.71 $\pm$ 0.35 d
	Low	120	21.81 $\pm$ 0.33 c	25.16 $\pm$ 0.64 ab	19.48 $\pm$ 0.52 b
	Low	180	25.35 $\pm$ 0.09 a	26.71 $\pm$ 1.48 a	20.69 $\pm$ 0.45 a
	High	0	18.19 $\pm$ 0.43 d	21.43 $\pm$ 0.35 d	18.26 $\pm$ 0.31 c
	High	120	23.70 $\pm$ 0.45 b	25.27 $\pm$ 1.03 b	19.58 $\pm$ 0.51 b
	High	180	21.53 $\pm$ 0.96 c	23.62 $\pm$ 0.53 c	18.63 $\pm$ 0.42 c
2018	Low	0	19.58 $\pm$ 0.45 d	23.81 $\pm$ 0.22 c	18.37 $\pm$ 0.24 b
	Low	120	23.70 $\pm$ 0.35 b	27.37 $\pm$ 0.13 a	20.62 $\pm$ 0.75 a
	Low	180	26.00 $\pm$ 0.71 a	27.66 $\pm$ 0.45 a	21.58 $\pm$ 0.25 a
	High	0	19.31 $\pm$ 0.29 d	21.85 $\pm$ 0.67 c	19.08 $\pm$ 0.26 b
	High	120	24.56 $\pm$ 0.19 b	25.59 $\pm$ 0.71 b	20.62 $\pm$ 1.23 a
	High	180	21.93 $\pm$ 0.83 c	26.00 $\pm$ 0.64 b	18.97 $\pm$ 0.64 b
2019	Low	0	18.72 $\pm$ 0.54 e	22.42 $\pm$ 0.31 c	17.10 $\pm$ 0.62 c
	Low	120	22.58 $\pm$ 0.21 c	25.00 $\pm$ 0.50 ab	19.22 $\pm$ 0.50 ab
	Low	180	25.30 $\pm$ 0.64 a	25.80 $\pm$ 0.56 a	20.10 $\pm$ 0.85 a
	High	0	17.76 $\pm$ 0.47 e	20.98 $\pm$ 0.63 d	18.70 $\pm$ 0.14 b
	High	120	23.67 $\pm$ 0.71 b	25.35 $\pm$ 0.75 ab	19.28 $\pm$ 0.13 ab
	High	180	21.18 $\pm$ 0.76 d	24.24 $\pm$ 1.23 b	18.68 $\pm$ 0.38 b
ANOVA					
Year (Y)			***	***	***
Density (D)			***	***	*
Nitrogen (N)			***	***	***
Y $\times$ D			**	**	**
Y $\times$ N			**	ns	ns
D $\times$ N			***	***	***
Y $\times$ D $\times$ N			*	***	**

3.3. Effect of Nitrogen Application and Planting Density on Antioxidant Enzyme Activities

Table 4 shows the summary statistics of antioxidant enzyme activities under two density and three N applications across years. ANOVA results indicated that the differences among years and N applications, between densities, and variances due to interactions between any two factors (year, density, and N application) and among the three factors were significant or highly significant for most antioxidant enzyme activities. Under low-density treatment, the antioxidant enzyme activity increased with an increase of nitrogen application at $N_2 > N_1 > N_0$, and the performance was consistent over three years. Under the high-density treatment, with the increase of nitrogen, the antioxidant enzyme activity first increased then decreased, showing $N_1 > N_2 > N_0$, but higher than the N_0 treatment. Compared with no N application, SOD, CAT, POD, and APX activities increased by 19.51%, 17.39%, 73.77%, and 38.54% under the N_1 treatment, and increased by 19.37%, 15.93%, 68.53%, and 44.12% under the N_2 treatment, respectively. Under the treatment of nitrogen application, high density increased the activity of antioxidant enzymes. Compared with low-density planting, high-density planting led to decreased SOD, CAT, POD, and APX activities by 8.20%, 6.99%, 16.41%, and 14.28%, respectively. The results showed that excessive planting density reduced the activity of antioxidant enzymes, but that activity of antioxidant enzymes increased with N application.

Table 4. Effect of nitrogen application and planting density on antioxidant enzyme activities ($U \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$). Different lowercase letters after the same column of values represented significant differences ($p < 0.05$) in the same year according to LSD. Values are the mean $\pm$ standard error ($n = 3$). ns: denotes non-significance; *, and ***: denote significance at $p < 0.05$, and 0.001, respectively.

Year	Density	Nitrogen	SOD $U \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$	POD $U \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$	CAT $U \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$	APX $U \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$
2017	Low	0	337.55 $\pm$ 6.80 e	416.93 $\pm$ 11.28 b	176.41 $\pm$ 5.10 e	134.18 $\pm$ 2.06 d
	Low	120	407.23 $\pm$ 2.77 b	485.88 $\pm$ 16.63 a	314.50 $\pm$ 12.97 b	181.27 $\pm$ 3.74 b
	Low	180	430.91 $\pm$ 10.97 a	486.14 $\pm$ 18.52 a	338.41 $\pm$ 7.94 a	208.15 $\pm$ 14.24 a
	High	0	331.01 $\pm$ 5.02 e	395.37 $\pm$ 6.48 b	166.36 $\pm$ 7.51 e	118.09 $\pm$ 2.31 e
	High	120	393.13 $\pm$ 7.67 c	472.54 $\pm$ 1.86 a	288.42 $\pm$ 11.70 c	167.54 $\pm$ 4.93 c
	High	180	373.42 $\pm$ 2.61 d	437.22 $\pm$ 9.05 b	240.77 $\pm$ 5.77 d	158.87 $\pm$ 3.91 c
2018	Low	0	350.38 $\pm$ 11.43 c	416.42 $\pm$ 8.76 c	186.02 $\pm$ 7.23 e	134.19 $\pm$ 2.69 e
	Low	120	409.91 $\pm$ 9.36 a	471.00 $\pm$ 24.28 b	314.12 $\pm$ 7.69 b	184.76 $\pm$ 6.25 b
	Low	180	425.43 $\pm$ 7.78 a	510.87 $\pm$ 1.98 a	338.84 $\pm$ 15.02 a	205.86 $\pm$ 4.81 a
	High	0	322.17 $\pm$ 6.75 d	383.79 $\pm$ 10.7 d	165.13 $\pm$ 8.43 f	120.30 $\pm$ 3.92 f
	High	120	386.21 $\pm$ 2.93 b	458.7 $\pm$ 4.45 b	288.11 $\pm$ 13.53 c	169.19 $\pm$ 2.22 c
	High	180	364.55 $\pm$ 16.58 c	431.3 $\pm$ 13.53 c	251.80 $\pm$ 10.22 d	154.81 $\pm$ 2.52 d
2019	Low	0	335.69 $\pm$ 8.6 d	413.35 $\pm$ 17.65 b	177.93 $\pm$ 8.90 e	134.05 $\pm$ 0.44 d
	Low	120	410.59 $\pm$ 19.91 b	467.11 $\pm$ 3.39 a	317.27 $\pm$ 6.04 b	181.51 $\pm$ 5.78 b
	Low	180	438.80 $\pm$ 16.01 a	488.43 $\pm$ 19.31 a	340.43 $\pm$ 9.88 a	202.93 $\pm$ 2.98 a
	High	0	330.41 $\pm$ 4.64 d	380.68 $\pm$ 8.87 c	167.48 $\pm$ 5.37 e	119.68 $\pm$ 2.41 e
	High	120	391.74 $\pm$ 15.01 b	469.87 $\pm$ 12.01 a	283.64 $\pm$ 5.73 c	169.30 $\pm$ 4.30 c
	High	180	362.91 $\pm$ 17.22 c	435.98 $\pm$ 17.84 b	241.31 $\pm$ 4.39 d	165.40 $\pm$ 4.09 c
ANOVA						
Year (Y)			ns	*	*	ns
Density (D)			***	***	***	***
Nitrogen (N)			***	***	***	***
Y $\times$ D			ns	*	ns	*
Y $\times$ N			*	***	ns	ns
D $\times$ N			***	***	***	***
Y $\times$ D $\times$ N			*	*	***	ns

3.4. Effect of Nitrogen Application and Planting Density on N Use Efficiency

Table 5 shows summary statistics of N use efficiency related traits under two density and three N applications over years. ANOVA results indicated that the differences among

years and N applications between densities were significant or highly significant for most of N use efficiency related traits, while variances due to interactions between any two factors (year, density, and N application) and among the three factors were insignificant for most of N use efficiency related traits. Nitrogen application and planting density significantly affected rice REn, PFPn, IEn, and HIn. The interaction of nitrogen application by planting density significantly affected PFPn, Pen, and HIn across three years (Table 5). The impact on PFPn and HIn is significant. Compared with low-density planting, high-density planting led to a decrease in PFPn by 5.73%, and an increase in HIn by 1.32%. Compared with the N₁ treatment, the N₂ treatment decreased REn, AEn, and PFPn by 8.98%, 11.80%, and 25.14%, respectively. Compared with the N₀ treatment, the N₁ treatment increased HIn by 8.50%. There was no difference in HIn between N₀ and N₂. Increasing N application rate had no significant effect on PFPn in low-density planting but significantly decreased PFPn by 11.34% in high-density planting. HIn ranked as N₁ > N₂ > N₀ in low planting density and N₁ > N₀ > N₂ in high-density planting. The above results explained that excessive N application and high planting density reduced the N use efficiency, especially when both were excessive.

Table 5. Effect of nitrogen application and planting density on N use efficiency. Different lowercase letters after the same column of values represented significant differences ($p < 0.05$) in the same year according to LSD. Values are the mean $\pm$ standard error ($n = 3$). ns: denotes non-significance; *, ** and ***: denote significance at $p < 0.05$, 0.01 and 0.001, respectively.

Year	Density	Nitrogen	REn (%)	AEn (kg kg ⁻¹)	PFPn (kg kg ⁻¹)	PEn (kg kg ⁻¹)	IEn (kg kg ⁻¹)	HIn (%)
2017	Low	0						62.50 $\pm$ 2.04 d
	Low	120	32.36 $\pm$ 2.86 a	28.41 $\pm$ 2.44 a	65.18 $\pm$ 1.57 a	88.40 $\pm$ 12.24 a	101.73 $\pm$ 6.66 a	68.57 $\pm$ 0.32 b
	Low	180	30.90 $\pm$ 1.67 a	25.76 $\pm$ 3.33 a	50.27 $\pm$ 4.03 b	83.67 $\pm$ 12.98 a	96.50 $\pm$ 8.39 a	65.90 $\pm$ 0.86 c
	High	0						63.29 $\pm$ 1.00 d
	High	120	34.38 $\pm$ 1.71 a	26.54 $\pm$ 5.68 a	66.77 $\pm$ 3.41 a	76.78 $\pm$ 12.42 a	98.21 $\pm$ 3.33 a	69.21 $\pm$ 0.42 a
	High	180	30.92 $\pm$ 1.76 a	22.92 $\pm$ 3.23 a	49.74 $\pm$ 1.82 b	73.90 $\pm$ 6.11 a	93.30 $\pm$ 1.54 a	61.55 $\pm$ 1.86 d
2018	Low	0						63.11 $\pm$ 1.90 bc
	Low	120	35.16 $\pm$ 0.80 a	27.04 $\pm$ 3.53 a	61.55 $\pm$ 3.48 b	76.81 $\pm$ 8.40 a	90.23 $\pm$ 4.48 a	67.38 $\pm$ 1.85 a
	Low	180	30.62 $\pm$ 2.21 a	23.82 $\pm$ 4.82 a	46.82 $\pm$ 4.44 c	78.53 $\pm$ 19.28 a	89.23 $\pm$ 11.60 a	64.63 $\pm$ 1.16 b
	High	0						64.00 $\pm$ 0.53 b
	High	120	34.83 $\pm$ 3.18 a	28.59 $\pm$ 8.22 a	70.10 $\pm$ 4.89 a	84.12 $\pm$ 33.00 a	98.78 $\pm$ 9.74 a	68.89 $\pm$ 0.35 a
	High	180	33.45 $\pm$ 2.47 a	20.40 $\pm$ 3.12 a	48.08 $\pm$ 3.39 c	60.78 $\pm$ 4.90 a	83.41 $\pm$ 4.78 a	61.36 $\pm$ 1.36 c
2019	Low	0						63.62 $\pm$ 1.39 c
	Low	120	31.57 $\pm$ 4.20 ab	25.87 $\pm$ 7.10 a	57.59 $\pm$ 5.46 a	84.53 $\pm$ 32.85 a	90.68 $\pm$ 9.86 a	68.46 $\pm$ 1.50 a
	Low	180	30.03 $\pm$ 1.74 ab	25.70 $\pm$ 2.16 a	46.84 $\pm$ 0.69 a	85.57 $\pm$ 4.37 a	91.29 $\pm$ 4.08 a	66.17 $\pm$ 2.10 b
	High	0						63.94 $\pm$ 0.67 bc
	High	120	34.68 $\pm$ 2.00 a	25.6 $\pm$ 2.85 a	65.04 $\pm$ 5.43 a	73.75 $\pm$ 5.70 a	94.61 $\pm$ 7.76 a	70.29 $\pm$ 0.65 a
	High	180	28.84 $\pm$ 1.22 b	21.08 $\pm$ 1.91 a	47.38 $\pm$ 3.43 a	73.36 $\pm$ 9.51 a	92.06 $\pm$ 9.34 a	59.99 $\pm$ 0.52 d
ANOVA								
Year (Y)			***	ns	**	ns	***	**
Density (D)			ns	ns	***	**	ns	**
Nitrogen (N)			***	***	***	*	***	***
Y $\times$ D			ns	ns	**	ns	ns	*
Y $\times$ N			ns	ns	ns	ns	*	***
D $\times$ N			ns	ns	***	ns	ns	***
Y $\times$ D $\times$ N			***	ns	*	ns	***	ns

4. Discussion

The nitrogen fertilizer application rate, transplanting density, and their interaction were important factors affecting rice yield and nitrogen use efficiency [28–30]. To provide more data on the management of nitrogen fertilizer and transplanting density in rice production, we studied the effects of nitrogen fertilizer application, transplanting density, and their interaction on yield and its components, photosynthesis, antioxidant enzyme

activities, and nitrogen use efficiency. We found that increasing the transplanting density increased the effective panicle number and decreased the grain number per panicle and yield, and these findings are consistent with Berhanu et al. [16]. Higher transplanting density can guarantee the initial population number of rice, such as the number of tillers, but an excessive number of tillers will reduce the rate of panicle formation, resulting in waste of carbohydrates and water accumulation in ineffective tillers, which led to a reduced number of grains per panicle and yield [22]. With an increase of nitrogen application rate, the effective panicle number, grain number per panicle, and yield increased, which was consistent with findings reported by Tian et al. [26]. Nitrogen is one of the basic elements of plant growth. Thus, increasing its application rate can promote the development of the rice root system and the accumulation of dry matter above the ground and enhance the nutritional structure and higher yield [26].

We found that the net photosynthetic rate of rice at high density was lower than that at low density, which was consistent with the finding of Xu et al. [6]. Increasing the transplanting density also increased the shading rate between rice leaves and degraded the ventilation conditions among rice populations [31,32], thus resulting in a decrease in net photosynthetic rate. In addition, the increased planting density also decreased the nitrogen content that accumulated per panicle, and the nitrogen content was positively correlated with photosynthetic ability [33]. Nitrogen application increased the net photosynthetic rate at low density, but the net photosynthetic rate did not increase with the increase of nitrogen application at high density, consistent with the findings reported by Hou et al. [23]. Nitrogen promoted chlorophyll synthesis in rice and increased the net photosynthetic rate of leaves [26]. However, high nitrogen input increased the nutritional structure of rice, ultimately increasing the shading rate and reducing the ventilation rate among plants [26]. We found direct evidence that the photosynthetic rate at treatment N₁ was higher than at N₂ at low planting density but not at high density.

In this study, increasing the transplanting density decreased the antioxidant enzyme activity of rice. There are few previous reports on the effect of transplanting density on antioxidant enzyme activity. However, Wu et al. showed that increasing the transplanting density would increase the risk of disease infecting rice and affect the redox system [33]. The risk of disease and insect pests caused by close planting may cause environmental stress on rice and alter the activity of antioxidant enzymes. High planting density reduced the carbohydrate accumulation per panicle, and a lack of nutrition may also be part of the reason for the decrease of antioxidant enzymes in rice. The antioxidant enzyme activity of treatment N₂ was greater than that of N₁ at low planting density, but opposite results were observed at a high planting density. Nitrogen application increased the activity of antioxidant enzymes, which was consistent with the conclusion of Zhong et al. [34]. Nitrogen can support the normal growth of plants and maintain the oxidation balance in plants [35,36]. In addition, some studies have found that nitrate consumption contributes to a higher ATP level in cells under stress, and the conversion of NO₃[−] to NH₄⁺ is the main way to maintain a pH steady-state in plants under hypoxia [37]. This exhibited that nitrogen contributed to the normal oxidation balance in plants, reducing the production of free radicals and reactive oxygen species (ROS) as well as the consumption of antioxidant enzymes.

High density reduced PFPn, which was similar to findings reported by Lu et al. [38]. However, there were differences between our conclusions and those of that study, which may be because high planting density reduced rice yield (Table 2) and increased HIn, which was consistent with the conclusion of Xie et al. [27]. Increasing the planting density may accelerate the efficiency of N transport from stem and leaf to grain, and then increase the content of N in grain [27]. Increasing nitrogen application rates from level N₁ to N₂ decreased REN, AEn, and PFPn, which was consistent with the conclusion of Wang et al. [39]. This showed that there is a threshold for the nitrogen demand of plants, and excessive application of nitrogen fertilizer cannot be absorbed by plants, which may remain in the soil or be lost to the surrounding environment. Compared with treatment N₀, only N₁ increased HIn, which was consistent with the findings of Pan et al. [40]. These results

indicated that increasing the nitrogen application rate reduced the transport of N from stem and leaf to the grain. High planting density decreased PFPn and HIn, proving that excessive nitrogen fertilizer was not absorbed or utilized by plants.

In the present study, we found that most tested traits were affected by environment, planting density, N application, and their interactions. Fortunately, the annual results showed consistent trends at different nitrogen applications and planting densities. To better understand the effects of N applications, planting density and their interaction on grain yield, nitrogen use efficiency, and antioxidant enzymes, it is necessary to further investigate these traits using more rice varieties across different ecological areas with diverse soils and climates.

5. Conclusions

We found that increasing planting density increased the effective panicle number but decreased the grain number per panicle, yield, net photosynthetic rate, antioxidant enzyme activity, PFPn, and HIn. Increasing the nitrogen application rate increased the effective panicle number, grain number per panicle, and yield, but this did not lead to an increase in net photosynthetic rate or antioxidant enzyme activity. REn, AEn, PFPn, and HIn decreased with the increase of nitrogen application rate. Most tested traits in this study showed significant differences over years, N applications, and between densities. The interactions between nitrogen fertilizer and transplanting density significantly affected the net photosynthetic rate, antioxidant enzyme activity, PFPn, and HIn. In general, increasing nitrogen application at low planting density increases the above indices, while increasing nitrogen application at high planting density does not have the same effect due to the waste of applied nitrogen.

Author Contributions: W.W. was the principal investigator who designed and implemented the research. C.S. and Q.X. oversaw the study and performed the statistical analysis. B.D. and S.Z. wrote the manuscript and provided guidance during experimentation. D.X. reviewed the final version of the manuscript before submission for peer review. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Innovation and Application of Key Technologies for Light Simplified Direct Sowing of Rice in the North of the Middle and Lower Reaches of the Yangtze River (2017YFD0301403-06).

Data Availability Statement: Not applicable.

Acknowledgments: We thank Jianlong Xu (Beijing, China) for his critical comments on an earlier version of the manuscript.

Conflicts of Interest: The authors declare no competing interests.

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