

Article

Improving Tea Quality by Balancing ROS and Antioxidant System through Appropriate Ammonium Nitrogen Application

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Abstract: Nitrogen is an important nutrient for the tea plant (*Camellia sinensis*), which profoundly affects the quality and value of tea. In this study, the variations of biochemical activities, antioxidant systems, and tea quality of two tea varieties under four levels of nitrogen fertilizers were analyzed to explore the responses of tea plants to nitrogen stress. The primary maximum photochemical efficiency of PSII (F_v/F_m), the photochemical quenching coefficient (qP) and the relative electron transport rate ($rETR$) decreased under nitrogen deficiency (ND) and high nitrogen treatments (HN) in tea plant. Meanwhile, the levels of reactive oxygen species (ROS) increased significantly under ND/HN treatments, and the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) increased under HN/ND treatments. The relative expressions of antioxidant biosynthesis enzyme genes (*CsSOD*, *CsPOD*, *CsCAT* and *CsAPX*) were up-regulated under ND/HN treatments. Furthermore, the change trend of total free amino acid content under ND/HN treatments showed that nitrogen stress was not conducive to the accumulation of free amino acid content in tea, which may be related to the increase in ROS. This study presents a potential approach to improve tea quality by balancing ROS and antioxidant systems.

Keywords: *Camellia sinensis*; nitrogen; chlorophyll fluorescence; antioxidant activity



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1. Introduction

Tea (*Camellia sinensis* L.) is one of the important economic crops for leaf use in the world [1–4]. Nitrogen is an essential nutrient for sustaining tea plant growth and development, and it is also a key constituent element of tea quality, along with amino acids, tea polyphenols and caffeine [5–7]. Ammonium nitrogen (NH_4^+-N) is the favorite nitrogen form of tea plant [8–10], and could promote the improvement of tea yield and quality. Nowadays, the excessive application and deficiency of nitrogen fertilizer in tea plantations is still common, which greatly affects the quality and economic benefits of tea, and negatively affects the sustainable development of the tea industry, but its misuse can cause a series of problems, such as water pollution, degradation of soil quality, and osmotic stress, which finally leads to the decline of tea quality and yield [11–15].

Plants respond to biotic and abiotic stresses with a series of physiological and biochemical responses [16,17]. As a typical abiotic stress, nitrogen stress can induce plant growth inhibition and serious morphological, metabolic and physiological abnormalities [18]. Plant photosynthesis, for instance, is extremely sensitive to nitrogen stress. Photoinhibition was initiated in PSI and PSII in response to nitrogen stress, leading to the inhibition of relative electron transfer rate ($rETR$), and the rapid accumulation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($\text{O}_2^{\bullet-}$), and hydroxyl ($\bullet\text{OH}$) radicals in plants [19]. Redox reactions of ROS resulted in toxicity due to oxidative stress conditions, led to lipid peroxidation and DNA damage, and formed a large amount of malondialdehyde (MDA) [20]. In plants, a complex and multi-level antioxidant system (AOS) network

maintains intracellular homeostasis. Plants improve their ability to scavenge ROS by increasing antioxidant enzyme activity, such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), in order to withstand the damage of stress to plant cells [21]. The content of MDA can not only represent the degree of membrane lipid peroxidation, but it also reflects the strength of tissue antioxidant capacity indirectly. The content of MDA and the activities of SOD, POD, CAT are usually used as evaluation indexes of plant stress resistance [22,23].

Nitrogen starvation and nitrogen excess have effects on a series of physiological and biochemical processes in plants. Nitrogen deficiency causes oxidative stress to plants [24]. It was found that the antioxidant activity is positively correlated with the contents of total flavonoids, GSH, GSSG, anthocyanins and ascorbic acid in *Labisia pumila* (Blume) [25]. In tea plants, the primary maximum photochemical efficiency of PSII (F_v/F_m) decreased under a nitrogen deficiency condition, and the main reason for the decline in CO₂ assimilation was the decrease in photosynthetic electron transfer capacity, chlorophyll content and Rubisco enzyme initial activity [26]. In addition, high nitrogen stress caused oxidative damage to *Tetraselmis cordiformis*, during which MDA content was increased significantly compared with the control, and its contents of chlorophyll a (Chl a), chlorophyll b (Chl b) and the PSII maximum quantum yield reduced [27]. The activity of SOD, CAT and POD, the contents of H₂O₂, MAD, reduced glutathione (GSH), and the GSH/(oxidized glutathione) GSSG ratio increased under high nitrogen conditions in *Solanum lycopersicum* seedlings [28]. Furthermore, phytohormones could reduce the damage caused to plants under high ammonium stress [29].

The long-term rational application of nitrogen fertilizer was conducive to reduce the soil pollution, improve the yield and quality of tea, which caused an increase in tea's economic benefits. In this study, the responses of photosynthesis, antioxidant systems and tea quality of tea plants to different nitrogen levels were analyzed, which grounds a potential approach to resolve the degradation of tea quality through antioxidant systems.

2. Materials and Methods

2.1. Site and Materials

Location: The varieties “Baojinghuangjin tea 1#” (HJ1) and “Fudingdabaicha” (FD) were planted in pots and grown over 10 years. The tea plants were grown in the same experimental field and under identical conditions at the Hunan Tea Research Institute of China (28°12′23.49″ N, 113°4′31.37″ E). The mean annual temperature is between 18.0 and 18.7 °C, ranging from 0 °C in January to 37 °C in July, and the mean annual rainfall is 1403.1 mm.

Materials and preparation: The selected soil was classified as red soil, which was developed from quaternary red clay. The soil was naturally dried, and after crushing and mixing, the soil was sieved with a 2 mm aperture. Each pot was filled with 14.5 kg soil, and 2 tea plant seedlings were planted in each pot. The contents of soil alkali hydrolyzable nitrogen, rapidly available phosphorus and rapidly available potassium were 97.40 mg·kg^{−1}, 35.37 mg·kg^{−1} and 98.87 mg·kg^{−1} respectively, while the soil pH was 4.96. The tea varieties selected in this research were HJ1 and FD; the HJ1 variety was identified with a higher nitrogen utilization efficiency (NUE), and the FD variety was grown widely with an average NUE [7].

2.2. Design and Procedure

Different levels of ammonium treatments were employed to study the responses of the biochemical activity and antioxidant systems of the tea plant, and tea quality, to nitrogen stress. The research started in January 2012 with a pot experiment. The four fertilization treatments of each variety were 0 g (Nitrogen Deficiency, ND), 11 g (Low Nitrogen, LN), 22 g (Appropriate Nitrogen, AN) and 33 g (High Nitrogen, HN) of (NH₄)₂SO₄ (Sinopharm Chemical Reagent Co., Ltd., cat# 10002918, Shanghai, China) per pot (N dosage was 0 kg·ha^{−1}, 150 kg·ha^{−1}, 300 kg·ha^{−1} and 450 kg·ha^{−1}, respectively) ($n = 4$ per group), a

commonly used range during farming, and four pots without $(\text{NH}_4)_2\text{SO}_4$ treatment were used as the experimental control. The treatments were conducted at the stage when the bud germinated to 1–2 mm long (in March 2020); four weeks after this point (in April 2020), the bud would spread into a mature leaf. The chlorophyll fluorescence parameters, the H_2O_2 , $\text{O}_2^{\bullet-}$, and MDA contents, the SOD, CAT, POD, and APX enzyme activities, and the contents of GSSG and GSH were determined in mature leaves.

The 5th leaves from the top of each branch were collected separately on the 28th day after treatment. Leaves were rapidly frozen in liquid nitrogen and stored at -80°C . Three replication experiments were performed.

2.3. Chlorophyll Fluorescence Determination

The chlorophyll fluorescence parameters in tea plants were measured with a portable chlorophyll fluorometer, PAM 2500 (Walz, Effeltrich, Germany). The mature leaves and tender leaves from annual shoots of each treatment group were randomly selected for a chlorophyll fluorescence test [1]. Before measurements, these leaves were pre-adapted in the dark for 30 min using leaf clips to ensure the chlorophyll fluorescence yield was fully quenched. Minimum fluorescence (F_0) and maximum fluorescence (F_m) were measured, and the maximum photochemical efficiency of PSII (F_v/F_m) was calculated as $(F_m - F_0)/F_m$.

2.4. Determination of Peroxide Characteristics

The MDA content was an indicator of lipid peroxidation in plant leaves. The 0.25 g fresh tea leaf samples were weighed accurately, and a certain amount of 10% TCA and a small amount of quartz sand were added to the samples and ground to homogenate after centrifugation at $3000 \times g$ for 10 min. Then, 1 mL centrifuged supernatant and 1 mL 0.6% thiobarbituric acid solution were incubated at 100°C for 15 min; the mixture was rapidly cooled to room temperature, followed by centrifugation at $3000 \times g$ for 10 min. The supernatant was taken to determine extinction at 532 nm, 600 nm and 450 nm.

The H_2O_2 content was determined by titanium sulfate colorimetry. Here, 0.15 g fresh tea leaf samples were grounded with 1 mL pre-cooled acetone, the homogenate was centrifuged at $8000 \times g$ for 10 min at 4°C , and the supernatants were collected. To initiate the reaction, 0.1 mL extracting solution, 0.1 mL titanium sulfate and 0.2 mL concentrated ammonia were mixed, after the precipitation was formed. Followed by centrifugation at $4000 \times g$ for 10 min at 4°C , the precipitation was washed repeatedly with acetone 3–5 times. Then, 1 mL $2 \text{ mol}\cdot\text{L}^{-1}$ sulfuric acid was added to wash the precipitate. After it was completely dissolved, absorbance was determined at 415 nm to measure H_2O_2 content.

The hydroxylamine oxidation method was employed to determine the superoxide anion. The leaf samples (0.15 g) were homogenized with 1 mL potassium phosphate buffer (pH 7.8), and centrifuged at $10,000 \times g$ for 20 min. Then, 0.5 mL phosphate buffer (pH 7.8) and 1 mL $1 \text{ mmol}\cdot\text{L}^{-1}$ hydroxylamine hydrochloride were added to 0.5 mL supernatant. The mixture was incubated at 25°C for 20 min. Then, 1 mL $17 \text{ mmol}\cdot\text{L}^{-1}$ sulfanilamide and 1 mL $7 \text{ mmol}\cdot\text{L}^{-1}$ α -amino-phenylsulfonic were added to the mixture for another 20 min of incubation at 25°C . The absorption of the reaction mixture was measured at 530 nm.

2.5. Extraction and Determination of ROS-Related Antioxidant Enzymes

Here, 0.15 g tea leaf samples were homogenized with 1 mL $0.2 \text{ mol}\cdot\text{L}^{-1}$ potassium phosphate buffer (pH 7.8), and centrifuged at $10,000 \times g$ for 20 min at 4°C . The supernatant was used to determine the activities of SOD, CAT, POD and APX using the reagent kit (Suzhou Keming Biotechnology Co., Ltd., Suzhou, China) according to the instruction. The absorbance of the SOD, POD, CAT and APX reaction mixture was measured at 450 nm, 470 nm, 240 nm, and 290 nm, respectively.

2.6. Extraction and determination of Non-Enzymatic Antioxidant

To determine the glutathione (GSH) and glutathione disulfide (GSSG) contents, 1 mL 5% meta-phosphoric acid was added to 0.15 g tea leaf tissue for homogenization. This was

then centrifuged at $8000 \times g$ for 10 min, and the supernatants were used to determine the contents of GSH and GSSG [30]. To initiate the reaction, 0.7 mL $100 \text{ mmol} \cdot \text{L}^{-1}$ phosphate buffer (pH 7.5) and 0.2 mL $1 \text{ mmol} \cdot \text{L}^{-1}$ 5,5-dithiobis (2-nitrobenzoic acid) were mixed. Next, 0.1 mL of the supernatant was added to the reaction mixture. The absorbance at 412 nm was monitored to analyze the GSH content.

To analyze the GSSG content, we added 5 μL 2-vinylpyridine to 0.1 mL of the supernatant and kept it at 37°C for 30 min to mask the GSH. Then, 100 μL of 12.5 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), 10 μL $50 \text{ U} \cdot \text{mL}^{-1}$ GR (containing $50 \text{ mmol} \cdot \text{L}^{-1}$ phosphate buffer (pH 7.5) and $2.5 \text{ mmol} \cdot \text{L}^{-1}$ EDTA) and 700 μL 10 mM NADPH were added to the reaction mixture. After the reactants were added, the absorbances of 30 s and 150 s were measured at 412 nm.

2.7. RNA Extraction and qRT-PCR Assay

Tea leaf samples were ground into powder using liquid nitrogen. Total RNA was extracted using a TIANGEN RNAprep Pure kit (TIANGEN, catalog # DP441). Total RNAs were used for synthesizing the cDNA with a Fast Quant RT kit (TIANGEN, catalog # KR106). The cDNA was used in the qRT-PCR assay with SuperReal PreMix Plus (TIANGEN, catalog # FP205) on the Bio-Rad CFX96 realtime detection system (Bio-Rad, Hercules, CA, USA). The relative gene expression analysis was repeated three times. The primer sequences used for the qRT-PCR are listed in Table S2. Reactions were performed as follows: 95°C for 15 min followed by 40 cycles at 95°C for 10 s, and 58°C for 30 s. *CsACTIN* was used as the reference for the qRT-PCR data analysis.

2.8. Free Amino Acids Determination

Free amino acids were determined as described by Li et al. [1]. Here, 0.2 g of freeze-dried tea shoots were ground into powder. We then added 4.5 mL deionized water into the samples, which were incubated for 15 min at 100°C . The homogenate was centrifuged at 6000 rpm for 10 min, and the residues were re-extracted once. The supernatants were merged and diluted with water to a volume of 10 mL. Before HPLC analysis, the supernatants were filtered through a $0.22 \mu\text{m}$ membrane.

Free amino acids were detected using an e2695 Waters HPLC system (Waters, Milford, MA, USA) according to the manufacturer's specifications. A Waters AccQ-Tag was used to as the reversed-phase HPLC column ($150 \text{ mm} \times 3.9 \text{ mm}$, $5 \mu\text{m}$). The detection wavelength was 248 nm. The mobile phase consisted of AccQ-Tag (1:10 v/v) (A) in water and acetonitrile (6:10) (B) in water, and a line-gradient was eluted for 45 min to detect the free amino acids. Then, we injected 10 μL filtrate into the HPLC system for analysis.

2.9. Statistical Analysis

One-way ANOVA was conducted to determine the effect of nitrogen level on the antioxidant system and tea quality. All data were statistically analyzed using SPSS 21.0 (USA) and DPS7.05 (China) software, and significant differences among different nitrogen fertilization treatments in the same variety were tested using the Duncan's new complex range method. Standard deviations were obtained from the variability in 3–4 replicates of data.

3. Results

3.1. Appropriate Nitrogen Increased the Chlorophyll Fluorescence Parameters in Tea Plant

Under abiotic stress, the photosynthetic system of plants is one of the main sites to produce ROS. The maximum photochemical efficiency (F_v/F_m) of PSII reflects the primary light energy conversion efficiency in the PSII reaction center [31,32]. We studied the effect of nitrogen on PSII in tea plant. Compared with nitrogen deficiency (ND), after nitrogen application treatment, the F_v/F_m , the photochemical quenching coefficient (qP) and the relative electron transport rate ($rETR$) of mature leaves increased in HJ1 and FD, and the increase was maximal under appropriate nitrogen conditions (AN) (Figure 1a–c). The

variations in these parameters in tender leaves (Figure 1d–f) were generally consistent with mature leaves. However, the qP and $rETR$ in HJ1 were significantly lower than those in FD, indicating that the carbon assimilation rate of HJ1 was weaker than that of FD in tender leaves.

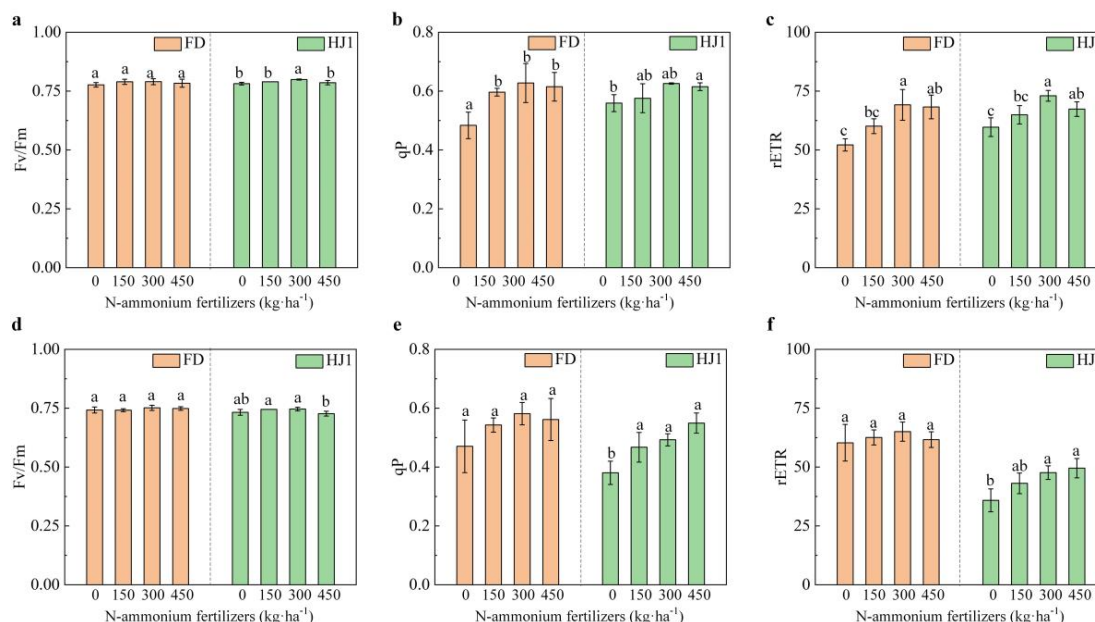


Figure 1. Effect of nitrogen on the chlorophyll fluorescence parameters of the leaves in tea plant. (a) F_v/F_m , (b) qP , (c) $rETR$ in mature leaves, (d) F_v/F_m , (e) qP , and (f) $rETR$ in tender leaves. The tea plants were tested using pure nitrogen 0 kg·ha^{−1} (ND), 150 kg·ha^{−1} (LN), 300 kg·ha^{−1} (AN) and 450 kg·ha^{−1} (HN). The values represented in (a–f) are the mean $\pm$ SD of 4 biological replicates ($n = 4$, Duncan's new complex range method). Different letters represent significant differences in the same tea variety ($p < 0.05$).

3.2. Appropriate Nitrogen Decreased the Peroxide Contents in Tea Plant

The H_2O_2 and $O_2^{\bullet-}$ contents are important indicators of oxidative damage under abiotic stress. Under the four nitrogen level conditions, the H_2O_2 content increased and the $O_2^{\bullet-}$ content increased significantly under ND in HJ1. The contents of H_2O_2 and $O_2^{\bullet-}$ were lower under low nitrogen (LN) and AN in FD and HJ1. Compared with ND, the content of $O_2^{\bullet-}$ decreased by 20.9% and 5.6% under AN in HJ1 and FD, respectively (Figure 2a,b).

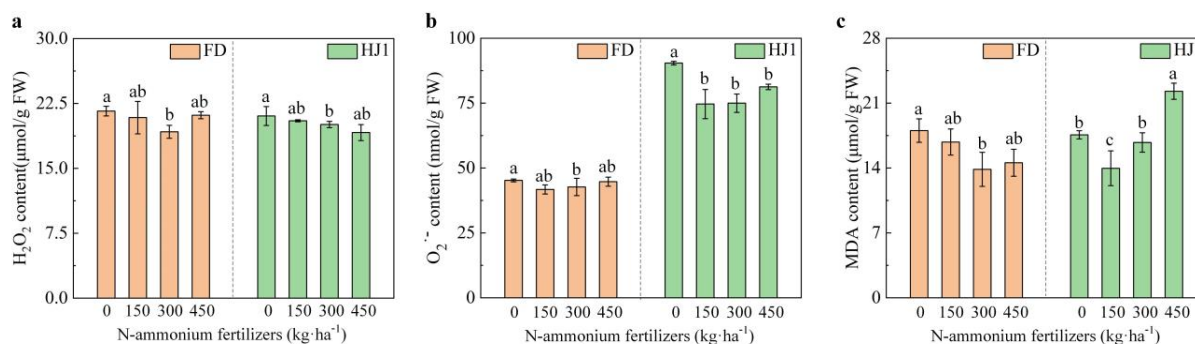


Figure 2. Effect of nitrogen on H_2O_2 , $O_2^{\bullet-}$ and MDA levels in tea leaves. (a) Malondialdehyde (MDA) content; (b) superoxide anions ($O_2^{\bullet-}$) content; (c) hydrogen peroxide (H_2O_2) content. Each data point represents the mean $\pm$ standard deviation (SD), $n = 3$. Different letters represent significant differences in the same tea variety ($p < 0.05$) as determined by Duncan's new complex range method.

Malondialdehyde (MDA) content is an important indicator of membrane structural integrity. In HJ1 and FD, the responses of MDA content to nitrogen application were different. Compared with the LN treatment in HJ1, the MDA content increased by 28.4% under the high nitrogen treatment (HN). Compared with AN in FD, the content of MDA was significantly increased by 23.3% under the ND treatment (Figure 2c).

3.3. Nitrogen Stress Affected the Activities of ROS-Related Antioxidant Enzymes in Tea Plant

Tea plant scavenges its excessive ROS under nitrogen stress through the AOS system. The changes in antioxidant enzyme activities under ND or HN conditions are one form of AOS. The superoxide dismutase (SOD) is directly involved in eliminating ROS. Compared with the other treatments, the SOD activity significantly increased under ND and HN treatments in FD and HJ1 (Figure 3a). The POD and APX activities were the highest under HN conditions in HJ1. Under the HN condition, the POD activities were 40.3% and 11.6% higher than those of the ND and AN treatments in HJ1, and the APX activity was higher than those under ND and AN treatments by 76.0% and 51.7% in HJ1. The CAT activity was significantly lower by 93.6% under the AN treatment than that of the ND treatment in HJ1. Compared with other nitrogen treatments, the activities of POD, CAT and APX were higher under ND and HN treatments in FD. The APX activity under AN treatment was lower than those of the ND and HN treatments by 53.1% and 37.5%, respectively, in FD (Figure 3b–d).

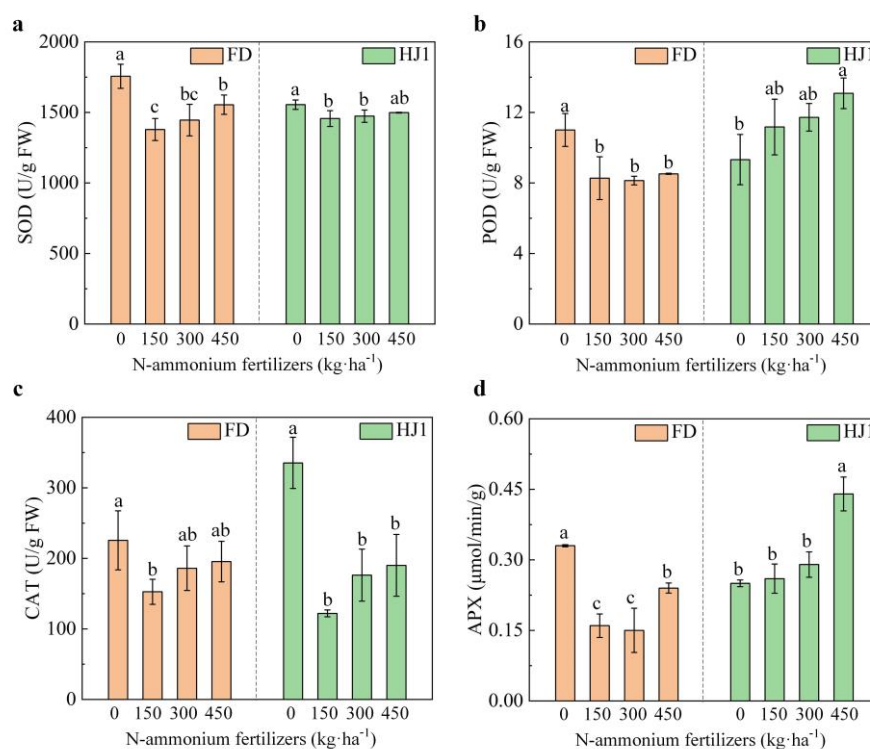


Figure 3. Effects of nitrogen on SOD, POD, CAT and APX enzymatic activities in tea leaves. (a) Superoxide dismutase (SOD); (b) peroxidase (POD); (c) catalase (CAT) and (d) ascorbate peroxidase (APX). Each data point represents the mean $\pm$ standard deviation (SD), $n = 3$. Different letters represent significant differences in the same tea variety ($p < 0.05$) as assessed by Duncan's new complex range method.

3.4. Nitrogen Affects the Contents of Non-Enzymatic Antioxidants in Tea Plants

As a part of AOS, GSH (glutathione) is one of the more important antioxidants in plant tissues. GSH is regenerated by GSSG (glutathione disulfide) in the oxidative state. Reduced glutathione GSH and its oxidized form GSSG were employed to scavenge ROS and maintain the redox balance in plant cells. As the nitrogen application rate increased, the GSSG content rose in tea cultivars, among which the GSSG content treated with HN

was significantly higher than that in the other nitrogen treatments (Figure 4). The GSSG contents of the two tea varieties under HN treatment were 94.7% and 67.9% higher than those under the ND treatment in FD and HJ1. Regarding GSH, its content with the LN treatment was lower than that of the ND and HN treatments in HJ1; their difference was not significant, however, compared with ND and HN, and the GSH content in FD was significantly decreased under AN treatment. Overall, the GSSG content of HJ1 under each treatment was 2.61–3.60 times that of FD, but the difference in GSH content was not as significant as that of GSSG between HJ1 and FD cultivars.

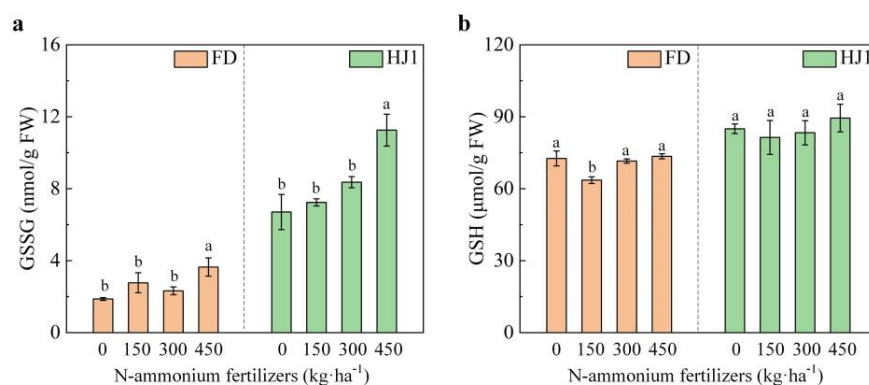


Figure 4. Effects of nitrogen on the GSH and GSSG contents in tea leaves. (a) Glutathione (GSH), (b) glutathione disulfide (GSSG). Each data point represents the mean $\pm$ standard deviation (SD), $n = 3$. Different letters represent significant differences in the same tea variety ($p < 0.05$) as assessed by Duncan's new complex range method.

3.5. Effects of Nitrogen on the Gene Expression of Antioxidant Enzymes in Tea Plant

To understand the molecular mechanism of nitrogen detoxification in abiotic stress in the tea plant, the relative expression levels of *CsSOD*, *CsPOD*, *CsCAT* and *CsAPX* were analyzed. Similar to the enzyme activities, the relative expressions of *CsSOD*, *CsPOD*, *CsCAT* and *CsAPX* with ND and HN were higher in FD, and AN was the lowest (Figure 5). The expressions of *CsCAT* and *CsAPX* genes were up-regulated after nitrogen treatment in HJ1, and the expressions of *CsCAT* and *CsAPX* in the HN treatment were 2.69 and 3.22 times than that in the ND treatment, respectively. The variations in the relative expressions of *CsSOD* and *CsPOD* in HJ1 were consistent with that in FD.

3.6. Appropriate Nitrogen Significantly Increased the Tea Quality

Nitrogen application significantly affected the accumulation of total free amino acids in tea plant (Table 1, Figure 6b). The total free amino acid contents of tea leaves under different nitrogen levels were analyzed. Compared with ND, the total free amino acids contents of HJ1 and FD significantly increased after nitrogen application. The highest increase in free amino acids was observed in the two tea cultivars under AN treatment, which indicates that AN treatment was conducive to the accumulation of nitrogen assimilation products in tea plant. In contrast, nitrogen deficiency stress cannot adequately supply the nutrient requirements of nitrogen metabolism in tea plant, and the key enzyme activity of nitrogen metabolism was affected significantly by nitrogen (ND/HN) stress, resulting in a decrease in total free amino acids (Figure 6b).

Table 1. Effect of nitrogen application level on the contents of total amino acids in tea leaves.

	ND (mg·g ⁻¹)	LN (mg·g ⁻¹)	AN (mg·g ⁻¹)	HN (mg·g ⁻¹)
FD	4.44 $\pm$ 0.01	4.77 $\pm$ 0.06 **	5.80 $\pm$ 0.07 **	5.16 $\pm$ 0.06 **
HJ1	4.59 $\pm$ 0.08	5.20 $\pm$ 0.06 **	6.69 $\pm$ 0.04 **	5.58 $\pm$ 0.05 **

** represents statistical t -test p values less than significance levels 0.01 under different nitrogen levels of the same variety.

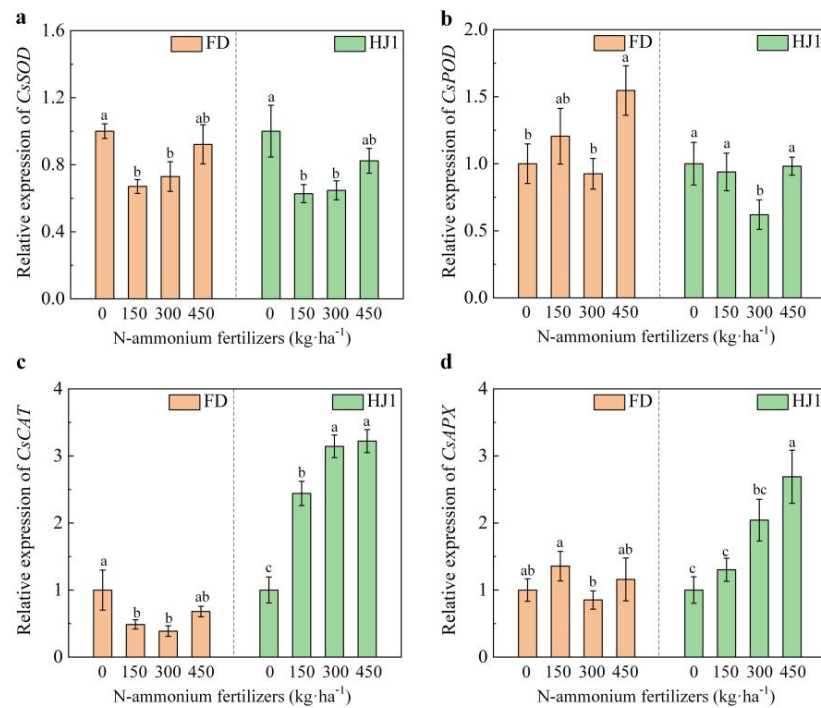


Figure 5. Effects of nitrogen on gene expression of antioxidant enzymes in tea leaves. (a) *CsSOD*, (b) *CsPOD*, (c) *CsCAT*, (d) *CsAPX*. Each data point represents the mean $\pm$ standard deviation (SD), $n = 3$. Different letters represent significant differences in the same tea variety ($p < 0.05$) as assessed by Duncan's new complex range method.

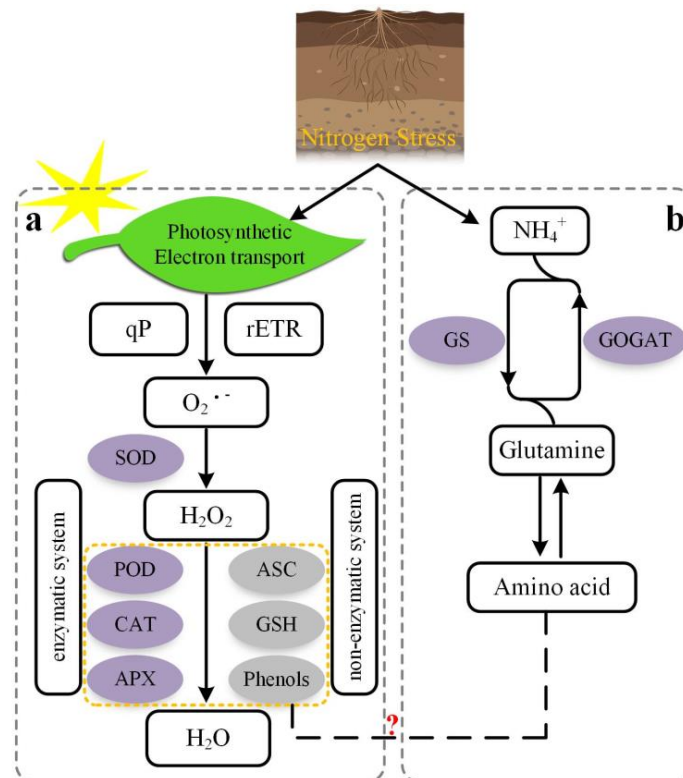


Figure 6. Response pathway of ROS and free amino acid content to nitrogen stress. (a) The metabolic pathways of ROS in tea plant under nitrogen stress; (b) the metabolic pathways of the amino acids tree under nitrogen stress in tea plant.

4. Discussion

Nitrogen is one of the most important mineral elements for the tea plant, and has a great impact on the yield and quality of tea [33,34]. Nitrogen excess or nitrogen deficiency affect the oxidative balance in tea plants [35], which directly inhibits the photosynthetic systems of plants [36–39]. In this study, the maximum photochemical efficiency (F_v/F_m) of PSII decreased under nitrogen deficiency (ND) and high nitrogen treatment (HN) (Figure 1a,d). The change trends of qP and $rETR$ were consistent with PSII, indicating that nitrogen stress had a great impact on the photosynthesis of the tea plant.

The inhibition of photosynthetic electron transport induced by nitrogen stress leads to the excessive accumulation of ROS, such as H_2O_2 and $O_2^{\bullet-}$ (Figures 2 and 6), which promotes the degradation of chlorophyll and reduces the photochemical effect of PSII [40,41]. Furthermore, the accumulation of ROS to a certain amount led to the lipid peroxidation of the cell membrane and the diminution of F_v/F_m . Its product MDA increased dramatically (Figure 2), resulting in the rapid aging of plants, causing the rapid senescence of the tea plant, which has been similarly reported in other crops such as rice and maize [15,42]. These results suggest that nitrogen stress (ND/HN) induced the production of H_2O_2 and $O_2^{\bullet-}$, and damaged the photosynthetic machinery [43], leading to disturbances in the physiological metabolism of the tea plant and the reduction of tea quality.

In general, plants improve their ability to scavenge ROS by increasing antioxidant enzyme activities when they suffer abiotic stress, in order to defend against the damage caused by stress to the plant cell membrane. H_2O_2 , as one of the main components of ROS, is a signaling factor that induces plants to defend against abiotic stresses [30,44]. Plants employ several strategies to scavenge ROS, including increasing the activity of antioxidant enzymes such as SOD, POD, CAT and APX, as well as regulating the contents of non-enzymatic antioxidants [21,45,46]. SOD acts as the first level of protection against ROS, transforming $O_2^{\bullet-}$ into H_2O_2 , APX, GPx, and CAT to further detoxify the resulting H_2O_2 (Figure 6a) [47]. In this study, the enzyme activities of SOD, POD, CAT and APX were increased under nitrogen deficiency and high nitrogen levels in FD and HJ1 (Figure 3). The variations in SOD and CAT activities were consistent with each other, both in HJ1 and FD. However, the POD and APX activities were higher under HN conditions in HJ1, which contrasts with the changes in FD, indicating that HJ1 has strong tolerance to high nitrogen stress, while FD had strong tolerance to low nitrogen stress [7,48]. This deduction was confirmed by the results of H_2O_2 and $O_2^{\bullet-}$ contents in FD and HJ1 under nitrogen treatments (Figure 2), which are consistent with our previous study as well [7]. At the same time, the up-regulation of the relative expressions of these genes (*CsSOD*, *CsPOD*, *CsCAT*, *CsAPX*) was directly involved in the increased enzyme activities [17,47,49]. Furthermore, as one of the most important antioxidants in plant tissues, GSH/GSSG is essential in maintaining redox homeostasis [50,51]. The GSH/GSSG variation trends of FD and HJ1 remained consistent under nitrogen stress; however, their absolute contents in FD and HJ1 showed differences. The GSH content showed a significant increase under high nitrogen stress in HJ1; on the contrary, the contents of GSH of FD significantly increased under nitrogen deficiency stress, consistent with the change trends of H_2O_2 and $O_2^{\bullet-}$ in FD, indicating that the tolerances of HJ1 and FD to nitrogen stress were quite different.

Nitrogen excessive or insufficient applications in the tea plantation caused abiotic stress to the tea plant, resulting in an increase in ROS content as well as the antioxidant defense system, which is not conducive to the growth and development of the tea plant, leading to a decrease in the total free amino acid (Table 1, Figure 6) [48]. Therefore, appropriate amounts of nitrogen fertilization applied to tea cultivars with different nitrogen tolerance capacities is beneficial for maintaining the optimal amount of ROS within the cellular environment, as well as the free amino acid accumulation and growth of the tea plant (Table S1). However, how the increased ROS under nitrogen stress affected the accumulation of amino acids in tea is not clear in the present study, which deserves further investigation.

5. Conclusions

In this study, we investigated the responses of the tea antioxidant system to nitrogen stress and its effect on tea quality. Under the conditions of nitrogen deficiency and high nitrogen, the chlorophyll fluorescence parameters F_v/F_m , qP and $rETR$ decreased, leading to the rapid increase in ROS content in the tea plant. The antioxidant defense systems of the tea plant were enhanced to scavenge the excessive ROS. The high nitrogen treatment had a greater impact on HJ1. Under HN conditions, the POD and CAT enzyme activities in HJ1 were 11.6% and 51.7% higher than those of the AN treatment, respectively. Meanwhile, it was found that the increasing of nitrogen within a certain range (ND-AN) was beneficial to promote the content of total free amino acids in tea, but excessive nitrogen application (HN) had negative effects on it. Under the conditions of AN, the physiological indexes were relatively balanced, which was conducive to the growth of tea and the improvement of tea quality, as well as the sustainable development of the tea plantation environment. Furthermore, the above results also show that the stress resistance among tea varieties was different, which provides a technical reference for developing fertilization regimes based on the specific targets of tea varieties in tea production.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/su14159354/s1>, Table S1: Effect of nitrogen application on the growth of different tea varieties. Table S2: Primers used for qRT-PCR assays in tea leaves.

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