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Effect of Nitrogen Fertilizer on Capsaicinoids and Related Metabolic Substances of Dried Chili Pepper Fruit

Chenfei Zhang [1,2], Lingfeng Shen [1,2], Shasha Yang [1,2], Tian Chang [1,2], Maolin Luo [1,2], Shanashan Zhen [1,2] and Xuehua Ji [1,2,*]

[1] Department of Horticulture, College of Agriculture, Shihezi University, Shihezi 832003, China; zchenfei0630@163.com (C.Z.); jokerdose@163.com (L.S.); daylight00@163.com (S.Y.); changtian0901@163.com (T.C.); luomaolin1202@163.com (M.L.); zss11062001@163.com (S.Z.)
[2] Key Laboratory of Physiology and Germplasm Resources Utilization of Special Fruits and Vegetables, Shihezi 832003, China
* Correspondence: lilysnowjxh@163.com

Abstract: Reducing fertilizer pollution is an important direction for modern ecological agriculture. Commonly, excessive nitrogen is applied to pepper. Capsaicin is one of the most important economic qualities of spicy peppers, but the effect of nitrogen on capsaicin is still inconsistent. This study aims to elucidate the impact of nitrogen fertilizer on capsaicin accumulation and to provide guidance on fertilizer application on highly spicy chili peppers. The experiment was conducted with five nitrogen fertilizer concentrations: N1 (urea 750 kg ha^{-1}), N2 (urea 562.5 kg ha^{-1}), N3 (urea 375 kg ha^{-1}), N4 (urea 187.5 kg ha^{-1}), and N0 (no nitrogen fertilizer). Nitrogen treatment was applied to two varieties with different spiciness levels from the seedling stage. The fruits in different layers of pepper plants were sampled on the 20th, 35th, and 50th day after anthesis, and the fruits' size, content of capsaicin, capsaicin precursors, capsaicin competitors, as well as capsaicin-related enzyme activities and gene expression level were analyzed. The results indicate that, when applying N2 and N3, both chili pepper varieties exhibited higher fruit length, diameter, weight, and yield values. There were increased contents of total phenol, flavonoids, and tannins in both fruit varieties with N2 application. Moreover, the placenta weights of the bottom, middle, and top layers of the fruits on the 35th day were improved by 40.14%, 26.80%, and 55.91% for 'Honglong 23' and 55.10%, 37.04%, and 75.56% for 'Hongxi' compared with N0. At the same time, under N2 treatment, the phenylalanine ammonia-lyase (PAL) enzyme activity of capsaicin synthase significantly increased. In contrast, the capsaicin-degrading enzyme activities of peroxidase (POD) and polyphenol oxidase (PPO) decreased notably. The expression levels of capsaicin-synthetic genes such as *phenylalanine cleavage enzyme* gene (*PAL*), *acyltransferase* gene (*AT3*), *4-Coumaroyl coenzyme A ligase* gene (*4CL*), *cinnamate 4-hydroxylase* gene (*C4H*), *caffeoyl coenzyme A-3-oxo-methyltransferase* gene (*COMT*), *paminotransferase* gene (*PAMT*), and *hydroxycinnamyltransferase* gene (*HCT*) were up-regulated in N2 fruits, which led to a significant increase in capsaicin content compared with the other four nitrogen amounts. A further reduction in nitrogen application to N3 and N4 resulted in a decrease in the precursor substance's total phenol content and PAL activity and an increase in the competitive substance's flavonoid, lignin, POD, and PPO enzyme activities. At the same time, the expression levels of capsaicinoid synthetic genes were downregulated in the N3 and N4 treatments, leading to a low content of total capsaicinoids. The capsaicinoid content showed a trend of 35th day > 50th day > 20th day for both varieties. Additionally, the contents of total capsaicinoids, total phenols, flavonoids, and lignins, as well as PAL enzyme activity, and the expression levels of *PAL*, *AT3*, *4CL*, *C4H*, *COMT*, *PAMT*, and *HCT* exhibited characteristics of bottom layers > middle layers > top layers. The activities of POD and PPO gradually increased from the bottom to the top layers. The N2 (562.5 kg ha^{-1}) treatment resulted in increases in placenta mass, maximum capsaicinoid precursor substance of total phenol content, and synthase enzyme activity, as well as decreases in capsaicinoid competing substances and degradative enzyme activity, so there were more substances available for capsaicin synthesis. Combined with the higher fruit weight and capsaicinoid content in the N2 treatment, N2 was considered a suitable nitrogen fertilizer dosage for highly spicy chili pepper cultivation.

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1. Introduction

Chili peppers (*Capsicum annuum* L.) are popular due to their unique taste, aroma, and color [1]. They have been widely used in food processing as flavoring and coloring agents [2]. Additionally, the fruits of chili peppers are rich in various bioactive substances and vitamins and have certain antioxidant properties [3,4], so chili peppers are the most valuable vegetables. The genus Capsicum covers 30 different species, five of which (*C. annuum* L., *C. chinensis* Jacq., *C. frutescens* L., *C. baccatum* L., and *C. pubescens* Ruiz and Pavon) have been successfully domesticated, and these have become one of the most popular food products consumed and grown globally [5]. According to FAO statistics from 2000 to 2010, more than 30% of the world's chili peppers are grown in China, and, as of 2021, China has become the largest producer of chili peppers. Industry chili peppers are categorized into dried and fresh forms, including whole dried, ground, fresh, or frozen chili peppers [6].

Capsaicin is the key substance that produces the pungent flavor of chili peppers [7]. Capsaicinoids have a variety of functions, such as anti-inflammatory [8], anticancer [9], pain relief [10], and antioxidant activities [11]. Capsaicinoids are a class of alkaloids mainly composed of capsaicin, dihydrocapsaicin, nordihydrocapsaicin, hypercapsaicin, and hyperdihydrocapsaicin. Capsaicin and dihydrocapsaicin account for 80–90% of the total capsaicinoids [12]. The accumulation of capsaicinoids generally begins in the epidermal cells of the placenta. Different parts of the mature pepper fruit, such as the placenta, pulp, and seeds, contain capsaicinoids, with the highest content in the placenta [13]. The metabolic pathway of capsaicin has been better defined, primarily formed by the condensation of vanillylamine with C9–C11 fatty acid fragments. A series of enzymes catalyze phenylalanine to derive vanillylamine, and valine is catalyzed by a series of enzymes to derive the branched-chain fatty acids of 8-methyl-6-nonenyl-coenzyme A. The two are catalyzed by capsaicin synthase (CS) to form capsaicinoids [14], which in turn produce a spicy taste [15].

Phenolic substances in plants are precursors of flavonoids, lignans, tannins, and capsaicinoids. Flavonoids are mainly synthesized through the phenylpropane metabolic pathway, for which phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (CA4H), and ligase of 4-coumarate CoA (4CL) are the key enzymes, both for this pathway and for capsaicin synthesis. The biosynthetic processes of lignin and tannin are also initiated by phenylalanine. Thus, capsaicin, flavonoid, lignin, and tannin synthesis share common substrates, and they have a comparative relationship to some extent. It has been found that the higher the maturity of chili pepper fruits, the higher the capsaicin content, but the accumulation of flavonoids declines [16]. Estrada et al. [17] have found that, in mature chili pepper fruits, the capsaicin content was negatively correlated with the POD and lignin content. This study has shown that the capsaicin content of pepper fruits increases with fertilization application, while phenolic compounds and lignin contents decrease significantly [18]. It was found that the expression levels of capsaicin synthetic genes, such as *C4H*, *COMT*, *KAS*, *PAMT*, and *AT3*, in the placenta of chili peppers were highly consistent with the capsaicin content [19].

Nitrogen is the most critical nutrient for plant growth and yield. Excessive or inappropriate use of nitrogen fertilizer not only leads to fertilizer waste but also triggers environmental pollution [20]. In general, excessive nitrogen causes spindling of the aboveground parts, thus delaying the maturity of fruits. When nitrogen is deficient, the bottom leaves turn yellow, and the new leaves become smaller.

Peppers are vegetables with long synchronized terms of blooming and fruit set, so peppers have a high requirement for nitrogen fertilizer. Aminifard et al. [21] have found that nitrogen application significantly increased the fruits' number and yield.

Nitrogen and potassium rationing significantly increased capsaicin content in fruits [22]. Wei et al. [23] considered that nitrogen, phosphorus, and potassium fertilizers on capsaicin potency were ranked as N > P_2O_5 > K_2O. With regard to ammonium (NH_4^+) and nitrate (NO_3^-)—two types of inorganic nitrogen that are necessary for the growth of plants—it was found that the growth, development and contents of capsaicin and dihydrocapsaicin of chili peppers increased when the ratio of NH_4^+:NO_3^- was 25:75 [24]. Johnson et al. [25] have found that capsaicinoid content in fruits was maximum at a nitrogen fertilization level of 15 mmol L^{-1}, and capsaicinoid content declined when nitrogen fertilization concentration was above or below this. Similar results were obtained in the study of Soares et al. [26], who found that, under nitrogen-deficient conditions, capsaicin and dihydrocapsaicin were minimal in the chili pepper fruits. Tilen et al. [27] found that capsaicin content was strongly associated with nitrogen content.

As mentioned above, the effect of nitrogen on capsaicin is still inconsistent. The high capsaicin content is dried chili peppers' most important economic characteristic. However, there are few studies about the optimal nitrogen fertilizer concentration for the spiciness of dried chili peppers, which is adverse to the improvement of the spiciness of chili peppers through cultivation measures. The experiment was carried out to screen the optimal nitrogen fertilizer concentration for maximizing capsaicin accumulation. This study compared the capsaicinoid content of the pepper fruit treated with different amounts of nitrogen fertilizer. It analyzed the changes in capsaicinoid precursor and competitive substances, capsaicinoid enzyme activities, and capsaicinoid-related gene expression levels to elucidate the influence of nitrogen fertilizer on pepper fruit spiciness. This study will provide a reference for fertilizer application of spicy peppers.

2. Materials and Methods

2.1. Overview of the Test Site

The experimental site was located in the Experimental Station of the College of Agriculture, Shihezi University, which is situated in the northern part of the Xinjiang province, within the range of 43°26′~45°20′ N latitude and 84°58′~86°24′ E longitude. The annual sunshine duration is 2721~2818 h, the annual average temperature ranges from 2 to 15 °C, the cumulative temperature $\geq$ 10 °C is between 3570 and 3729 °C, and the frost-free period is about 170 days. The sand soil's pH was 7.45, the organic matter content was 11.8 g kg^{-1}, the alkaline dissolved nitrogen content was 43.7 g kg^{-1}, the available potassium content was 155 mg kg^{-1}, and the available phosphorus content was 18.9 mg kg^{-1}.

2.2. Plant Material

Two dried chili pepper hybrids with different spiciness were selected: 'Honglong 23', which is a low-spice variety (spiciness 1000~2000 SHU), and 'Hongxi', which is a high-spice variety (15,000~30,000 SHU). Pepper seeds were provided by Xinjiang Tianjiao Hong'an Agricultural Science and Technology Limited Liability Company (Shihezi, China).

2.3. Fertilizer Treatment

Nitrogen fertilizer was urea (N $\geq$ 46%), phosphate and potassium fertilizer was potassium dihydrogen phosphate ($K_2O \geq 33.9\%$; $P_2O_5 \geq 51.5\%$), micronutrient fertilizer was formulated according to Hoagland Nutrient Solution, and the fertilizer was applied by drip irrigation under the mulch.

The experiment was conducted in the field in a one-row, two-hole manner. The pepper seedlings were transplanted on May 8 with 100 cm row space and 30 cm plant space. After transplantation, the seedlings (with 8~10 leaves) recovered 10 days before fertilizer treatment. Five nitrogen fertilizer gradients were set up: N1 (urea application 750 kg ha^{-1}), N2 (urea 562.5 kg ha^{-1}), N3 (urea 375 kg ha^{-1}), N4 (urea 187.5 kg ha^{-1}), and N0 (urea 0 kg ha^{-1}). Potassium dihydrogen phosphate dosage was 200 kg ha^{-1}, organic fertilizer dosage was 15,000 kg ha^{-1}, calcium magnesium sulfur dosage was 22.5 kg ha^{-1} and iron–manganese–copper–zinc concentration was 0.5%. Fertilizers were applied every

10 days for a total of eight times. The fertilizers were mixed with water for drip irrigation; 75% of the total fertilizer was applied over the first five times, and 25% was applied over the last three times. Each row was 12 m long, and the plot area of each treatment was 1.0 m × 12 m = 12 m^2; every treatment had three plot replications.

2.4. Indicator Measurement

2.4.1. Determination of Fruit Morphological Indicators

Samples were taken on the 20th day (fruit setting), 35th day (fruit expansion), and 50th day (red fruit) after anthesis; each treatment had five plant replications, and the fruits were picked from the top (1–2 layers of the pepper plant), middle (3–4 layers of the pepper plant), and bottom (5–6 layers of the pepper plant) of the plant. The length and diameter of the fruits were measured with vernier calipers, and the fruit shape index was calculated as the fruit length divided by the diameter. The fruit weight and placenta weight were determined with an analytical balance, and then the ratio of the placenta weight to fruit was calculated.

2.4.2. Determination of the Contents of Capsaicinoids and the Precursors and Competitive Substances

Capsaicin content was measured using high-performance liquid chromatography (LC-2010AHT) as described by Zhang et al. [28] and Li [29]. Total capsaicin content was calculated as (capsaicin content + dihydrocapsaicin content)/90%. Total phenols were determined using the Folin phenol method [30], flavonoids were determined using the aluminum ion colorimetric method [31], tannins were determined using the Folin–Denis colorimetric method [30], and lignin was determined using the acetylation method with [32] a kit provided by Suzhou Keming Biotechnology Co., Ltd. (Suzhou, China). The top, middle, and bottom layers of each treatment had three plant replicates. A total of 0.1 g of fruit flesh was used to measure tannin content. The remaining flesh was killed green at 105 °C for 20 min and dried at 75 °C to constant weight, then 0.1 g dried pulp was used to determine total phenol, flavonoid, and lignin content. The measuring instrument was a Microplate reader (SANYO, Tokyo, Japan). Total phenols and flavonoids determination, as follows: 0.1 g dried pulp was added to 2 mL extraction solution, shocked at 60 °C for 2 h, then centrifuged with 10,000× *g*, 25 °C for 10 min to obtain the supernatant. Tannin determination, as follows: 0.1 g dried pulp was added to 1 mL distilled water and placed in the water bath at 80 °C for 30 min, then centrifuged with 8000× *g* and 25 °C for 10 min. Lignin determination, as follows: 0.1 g dried pulp was added to 500 μL of reagent I, perchloric acid 20 μL, and a water bath for 40 min, then 500 μL of reagent II was placed into the tube, and 20 μL supernatant formed reagent III (1 mL). The tannins and total phenols were measured at 760 nm, the flavonoids at 510 nm, and the lignin at 280 nm.

2.4.3. Determination of Capsaicin-Related Enzyme Activity

Each treatment's top, middle, and bottom layers of fruits had three plant replicates. A total of 0.1 g of dried fruit pulp was ground with liquid nitrogen. The pulp was treated with the kit (provided by Suzhou Keming Biotechnology Co., Ltd.) (Suzhou, China), and the enzyme activities were measured by a microplate reader (SANYO). The PAL measuring process was undertaken as follows: 0.1 g dried pulp was added to 1 mL extraction which was centrifuged in an ice bath at 10,000× *g* and 4 °C for 10 min. POD and PPO were determined as follows: 0.1 g fresh pulp was added to 1 mL of extract and centrifuged in an ice bath at 8000× *g* and 4 °C for 10 min. PAL, POD, and PPO activities were measured at 290 nm, 470 nm, and 525 nm, respectively.

2.4.4. qRT-PCR

Each treatment's top, middle, and bottom layers of fruits had three plant replicates. RNA was extracted from fresh pepper pulp with an RNA kit (Xinjiang Kediyuan Biotechnology Co., Ltd.) (Shihezi, China). cDNA was synthesized using HyperScript III RT SuperMix for qPCR with gDNA remover (EnzyArtisan Biotech Co., Ltd.) (Shanghai, China). cDNA was synthesized using an SYBR Green Real-Time PCR Master Mix kit (EnzyArtisan Biotech Co., Ltd.). cDNA reaction system followed as total RNA (5 μg) + 8× gDNA remover (2 μL) + RNase-free ddH_2O (9 μL) + 5× RT Super Mix (4 μL). The cDNA reaction program was undertaken as follows: 42 °C 2 min, 50 °C 15 min, 85 °C 5 s. Quantitative PCR (qRT-PCR) was performed using an SYBR Green Real-Time PCR Master Mix kit (EnzyArtisan Biotech Co., Ltd.). qRT-PCR reaction system, as follows: 2× S6 Universal SYBR qPCR mix 5 μL + Primer-F 0.2 μL + Primer-R 0.2 μL + Template 1 μL + ddH_2O 3.6 μL. qRT-PCR reaction program, as follows: 5 °C, 30 s; 95 °C, 5 s; 60 °C, 30 s; 45 cycles at 72 °C and 20 s. Gene-specific primers were designed with Primer 5.0 software and synthesized by Xinjiang Youkang Biological Company (Xinjiang, China) with Actin as the internal reference gene. The primers are shown in Table 1. The relative expression level of genes was calculated using the ($2^{-\Delta\Delta Ct}$) method.

Table 1. qRT-PCR primer information of capsaicinoid-related genes.

Gene Name	Forward Primer	Reverse Primer
Actin (internal reference gene)	CACCCTGTCCTGCTCACTG	AAGAATGGCATGCGGCAAAG
PAL	CACAGTTTCAACATTACCCTTAGC	AAATGGTGGCAGAGTTTAGGAA
AT3	TTCCCATATAGCCCACTTGC	CAGCTCCCATATCGTTACAGTC
C4H	CTTTGGGACGTTTGGTGCAG	TCTCCAGAGCCCCTTAACTGA
4CL	CTTCTTCTCAACCATCCCAACA	ACGAAATCCTTGACTTCATCCTC
COMT	TAGCACATAACCCAGGAGGC	CACAGCACACCTTACGGAATCT
HCT	GTGTGGTGGAGTCTGCTTAGGT	GGTCAGTTGGTCGCTTGTGATC
PAMT	ATTGCCGCTGTCCTTGTA	CAGTTCCCCTTATCTCCCC

2.5. Data Analysis

Statistical analysis of variance (ANOVA) was performed using IBM SPSS Statistics 26.0 software, and relative expression levels of key genes were analyzed using Microsoft Excel 2013 (Microsoft, Redmond, WA, USA). Means were compared using Duncan's post hoc test at $p = 0.05$ and plotted using Origin 2021.

3. Results

3.1. Effect of Nitrogen Fertilizer on the Morphological Characteristics of Dried Chili Peppers Fruit

The fruit length (Figure 1A,B) and diameter (Figure 1C,D) of 'Honglong 23' were larger than those of 'Hongxi'. The length and diameter of the fruits of both varieties reached a maximum value on the 50th day after anthesis. The length and diameter of the fruit in the middle layer were higher than those of the top and bottom layers. The length and diameter of the fruits of the two varieties treated with nitrogen fertilizer were higher than those of N0. Fruit length values in the middle layers were higher than those in the top and bottom layers. The highest fruit lengths were found at the bottom layers of N3, which increased by 20.99% for 'Honglong 23' and 17.14% for 'Hongxi' when compared with N0 and by 5.64% for 'Honglong 23' and 12.33% for 'Hongxi' compared with N1. Fruit diameter was higher in fruits of the bottom layers than those of the middle and top layers, and the highest diameter was found at the bottom layers of N3, with an increase of 31.75% and 16.80% for 'Honglong 23' and 15.59% and 9.69% for 'Hongxi' when compared with N0 and N1, respectively. It can be inferred that, within a certain range of nitrogen amount, the length and diameter of pepper fruit increases with the increase in nitrogen application.

As shown in Figure 1E,F, in the bottom layers, the highest and the lowest fruit shape indexes were found at N2 and N3 of 'Honglong 23', respectively, while 'Hongxi' changed little with the nitrogen amount. In the middle layers, the highest and the lowest fruit shape index were found at N3 and N2 of 'Honglong 23' and N4 and N2 of 'Hongxi'. The maximum and minimum fruit shape indexes were found in the top layers of N1 of 'Honglong 23' and N3 and N1 of 'Hongxi', respectively. Appropriate nitrogen reduction could improve the shape index of the top layers of 'Hongxi'. The 'Honglong 23' fruit shape showed irregular changes in nitrogen amounts.

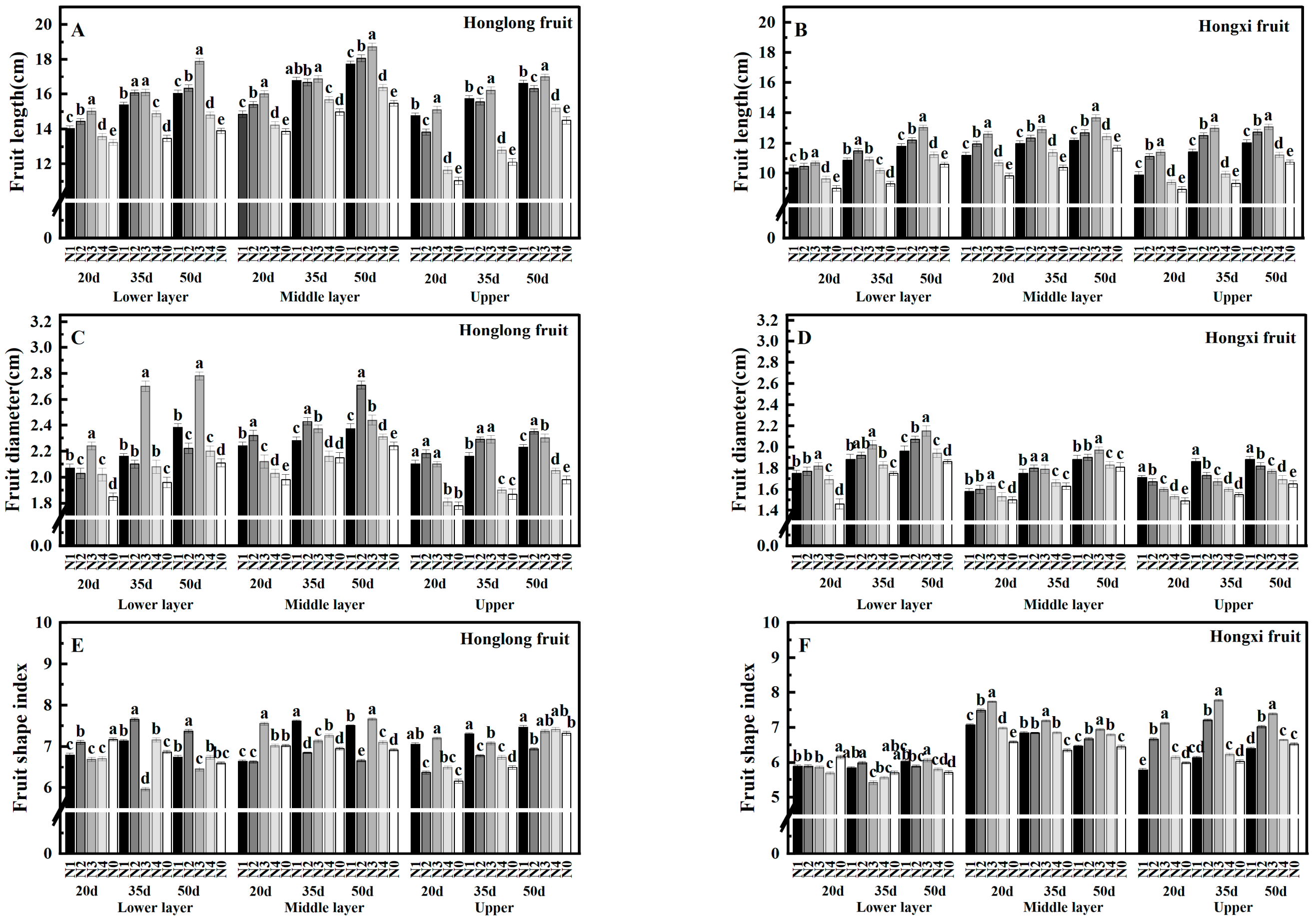

Figure 1. Effect of nitrogen fertilizer on fruit length (**A**,**B**), diameter (**C**,**D**), and the fruit shape of dried chili pepper (**E**,**F**). The data are presented as the mean ± standard error. Different lowercase letters indicate a significant difference among treatments according to the Duncan test ($p < 0.05$).

As shown in Figure 2A,B, 'Honglong 23' exhibited a greater fruit weight than 'Hongxi'. Both varieties displayed higher fruit weight in the middle layers. Maximum values were reached on the 50th day. The fruit weight of both peppers treated with nitrogen was greater than that of N0. Compared with N0, the fruit weight increased by 28.85% of N3 for 'Honglong 23' and 36.43% for 'Hongxi'. The fruit weights of the bottom of 'Honglong 23' and the top of 'Hongxi' were greater under the N2 treatment. The fruit weight of the middle and top layers of 'Honglong 23' and the middle layers of 'Hongxi' were higher under the N3 treatment. Overall, the fruit weight of 'Honglong 23' showed greater sensitivity to nitrogen fertilizer, whereas, in 'Hongxi' chili, the lower fruit weight was not affected by the treatments tested.

As depicted in Figure 2C,D, 'Honglong 23' had a heavier placenta weight than that of 'Hongxi'. The placenta weight of both varieties increased with fruit growth and reached its maximum on the 50th day after anthesis. The middle layers of fruit showed higher placenta weight in both varieties. The placenta weights of the treatments with nitrogen fertilizer were greater than those of N0. Higher placenta weight was found in the N2 treatment of different layers. On the 50th day, when compared with N0, placenta weight increased by 30.48% of N2 for 'Honglong 23' and 77.94% for 'Hongxi'. Similar to the fruit weight, the placenta weight of 'Honglong 23' exhibited greater sensitivity to nitrogen amount than that of 'Hongxi'.

As depicted in Figure 2E,F, the ratio of placenta weight to fruit weight of the two varieties treated with nitrogen was generally greater than those of N0. The placenta/fruit ratio of the bottom layers was higher under the N4 and N2 treatments, and lower under the N3 treatment of 'Honglong 23' and 'Hongxi', respectively. In the middle layers, the maximum values of the placenta/fruit ratio were found to be N2 and minimum values were found to be N3. The placenta/fruit ratio values were higher in the top layers under the N2 and lower under the N1 treatment.

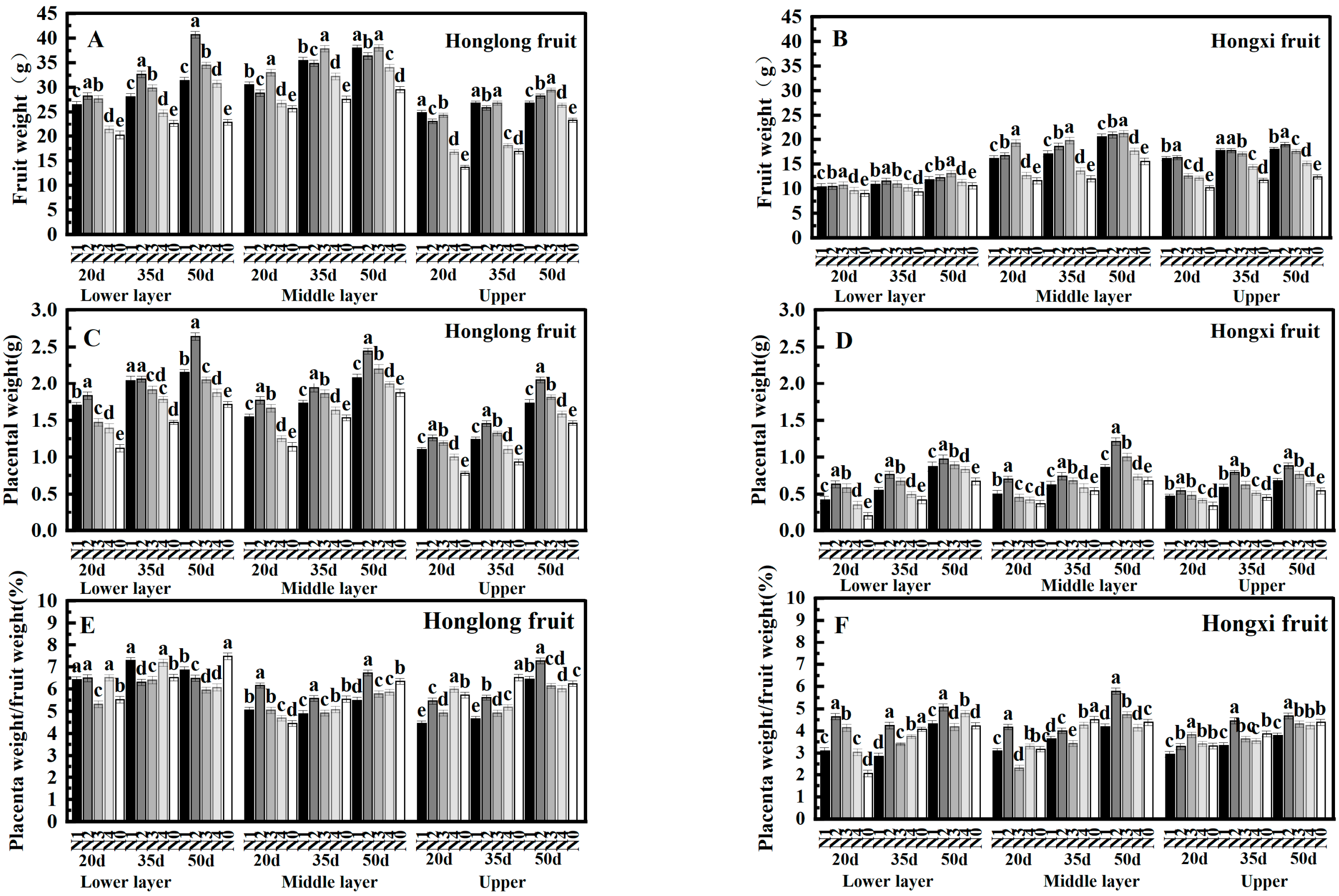

Figure 2. Effect of nitrogen fertilizer on fruit weight (**A**,**B**), placenta weight (**C**,**D**), and ratio of placenta weight/fruit weight of dried chili pepper (**E**,**F**). The data are presented as the mean ± standard error. Different lowercase letters indicate a significant difference among treatments according to the Duncan test ($p < 0.05$).

3.2. Effect of Nitrogen Fertilizer on the Capsaicin Content of Dried Chili Pepper Fruit

As shown in Figure 3, the capsaicinoid content of 'Hongxi' fruits was higher than that of 'Honglong 23', and the capsaicinoid contents of both varieties were ranked as follows: 35th day > 50th day > 20th day. The capsaicinoid contents of different layers of the two varieties followed the order of bottom layers > middle layers > top layers, indicating that capsaicinoid content decreased with the increasing fruiting position. Compared with N0, nitrogen application increased capsaicinoid accumulation, and the capsaicinoid contents were higher than N2 in three fruit development stages, followed by the N1 treatment. On the 35th day, compared with N0, this accumulation was increased by 160% of N2 at the bottom layers for 'Honglong 23' and 112.29% for 'Hongxi'. At the middle layers of N2, 'Honglong 23' was increased by 167.21% and 'Hongxi' was increased by 153.51%. At the top layers of N2, these were increased by 109.62% and 45.69%, respectively. Thus, in a certain range of nitrogen (N2, N3, N4, N0), capsaicin accumulation was positively related to the amount of nitrogen fertilizer.

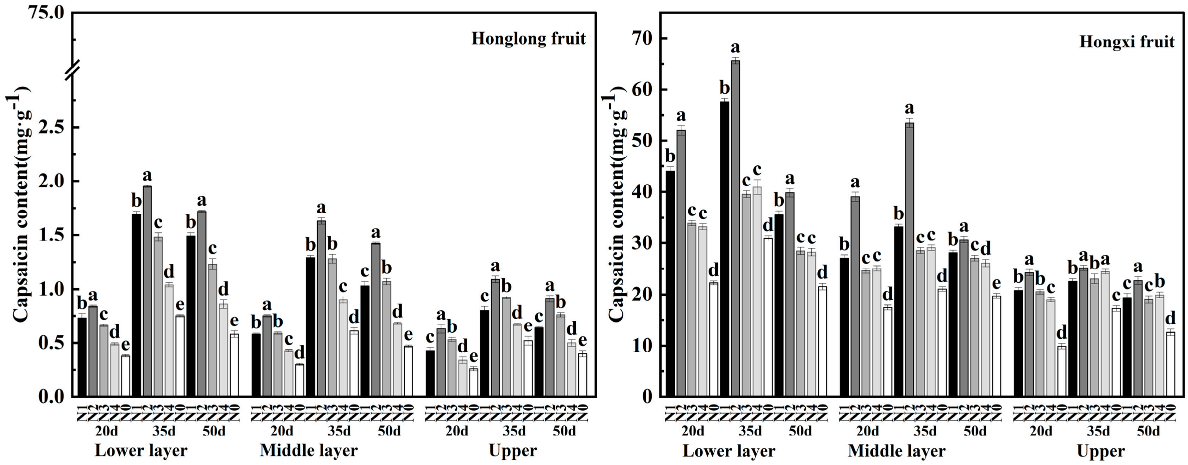

Figure 3. The effect of nitrogen fertilizer on capsaicin content of dried chili pepper. The data are presented as the mean ± standard error. Different lowercase letters indicate a significant difference among treatments according to the Duncan test ($p < 0.05$).

3.3. Effect of Nitrogen Fertilizer on the Precursor Substances of Capsaicin

As shown in Figure 4, the total phenolic content of 'Hongxi' was found to be more than two times higher than that of 'Honglong 23'. Both varieties' maximum total phenolic contents occurred on the 35th day, followed by the 50th day, consistent with the capsaicin content variation. For different layers, total phenolic content showed the following pattern: bottom layers > middle layers > top layers, which is consistent with the capsaicin contents of different fruit layers. Compared with N0, nitrogen application increased the accumulation of total phenols, and the highest total phenol content was found in the N2 treatment. On the 35th day, when compared with N0, at the bottom layers of N2, 'Honglong 23' and 'Hongxi' were increased by 28.90% and 33.28%, respectively; at the middle layers, these were increased by a respective 30.30% and 38.10%; finally, at the top layers, these were increased by a respective 38.21% and 52.48%. The above results indicate that, in a certain range, the accumulation of total phenols is positively related to the amount of nitrogen fertilizer.

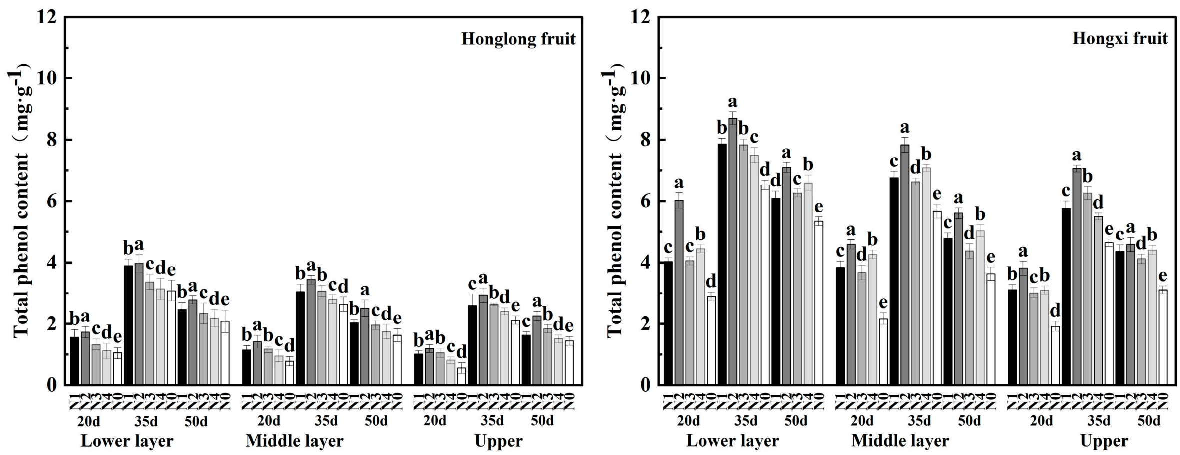

Figure 4. The effect of nitrogen fertilizer on the total phenolic content of dried chili pepper fruits. The data are presented as the mean ± standard error. Different lowercase letters indicate a significant difference among treatments according to the Duncan test ($p < 0.05$).

3.4. Effect of Nitrogen Fertilizer on the Competitive Substances of Capsaicin

As shown in Figure 5, the lignin and tannin contents of 'Hongxi' fruits were higher than those of 'Honglong 23', while the flavonoid contents were lower. The lignin, flavonoid, and tannin contents of the fruits of the two varieties were in the order of 50th day > 20th day > 35th day. The contents of the three capsaicinoid competitors of the two varieties were ranked as bottom layers > middle layers > top layers, which means that the competitive substances of capsaicin decreased with the upward shift of the fruit position. Compared with N0, nitrogen application increased the contents of lignin, flavonoid, and tannin in fruits. On the 50th day of treatment for the bottom layers treated with N2, when compared with N0, both lignin and flavonoids were increased by a respective 56.54% and 79.44% for 'Honglong 23'. Flavonoids and tannins were increased by a respective 183.55% and 31.29% for the 'Hongxi' treated with N3. The contents of the competitive substances of capsaicin were highest under the N2 and N3 treatments. It seems that the content of tannins in the fruit were positively related to the amount of nitrogen fertilizer to a certain extent(N3, N4, N0).

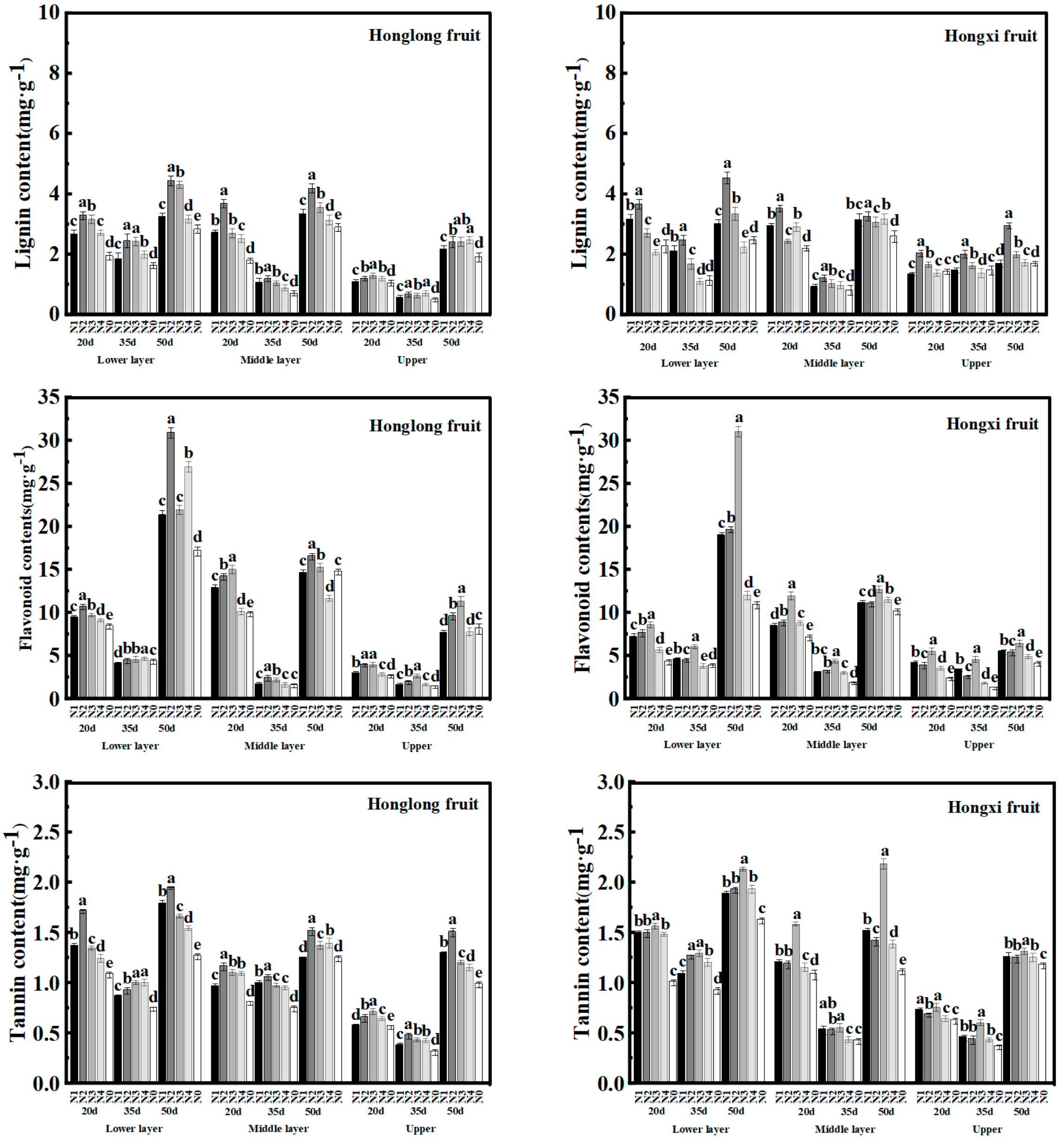

Figure 5. The effects of nitrogen fertilizer on the contents of lignin, flavonoids, and tannin of the dried chili pepper fruit. The data are presented as the mean ± standard error. Different lowercase letters indicate a significant difference among treatments according to the Duncan test ($p < 0.05$).

3.5. Effect of Nitrogen Fertilizer on the Activity of Capsaicinoid-Related Enzymes in Dried Chili Pepper Fruit

As shown in Figure 6, the PAL, POD, and PPO enzyme activities of 'Hongxi' were found to be higher than those of 'Honglong 23'. The PAL enzyme activities of both varieties showed the order as 35th day > 50th day > 20th day, while POD and PPO enzyme activities showed the order as 50th day > 20th day > 35th day. The PAL enzyme activities of different layers showed bottom layers > middle layers > top layers, while POD and PPO enzyme activities showed top layers > middle layers > bottom layers. Compared with N0, nitrogen application increased PAL, POD, and PPO enzyme activities, and treatments of N2 and N3 exhibited higher enzyme activities of PAL, PPO, and POD. On the 35th day of the bottom layers with N2, compared with N0, PAL enzyme activity was increased by 113.81% for 'Honglong 23' and 138.48% for 'Hongxi'. On the 50th day and for the top layers with N3, the POD, and PPO enzyme activities were increased by a respective 39.05% and 81.58% for 'Honglong 23', and 75.30% and 77.11% for 'Hongxi'.

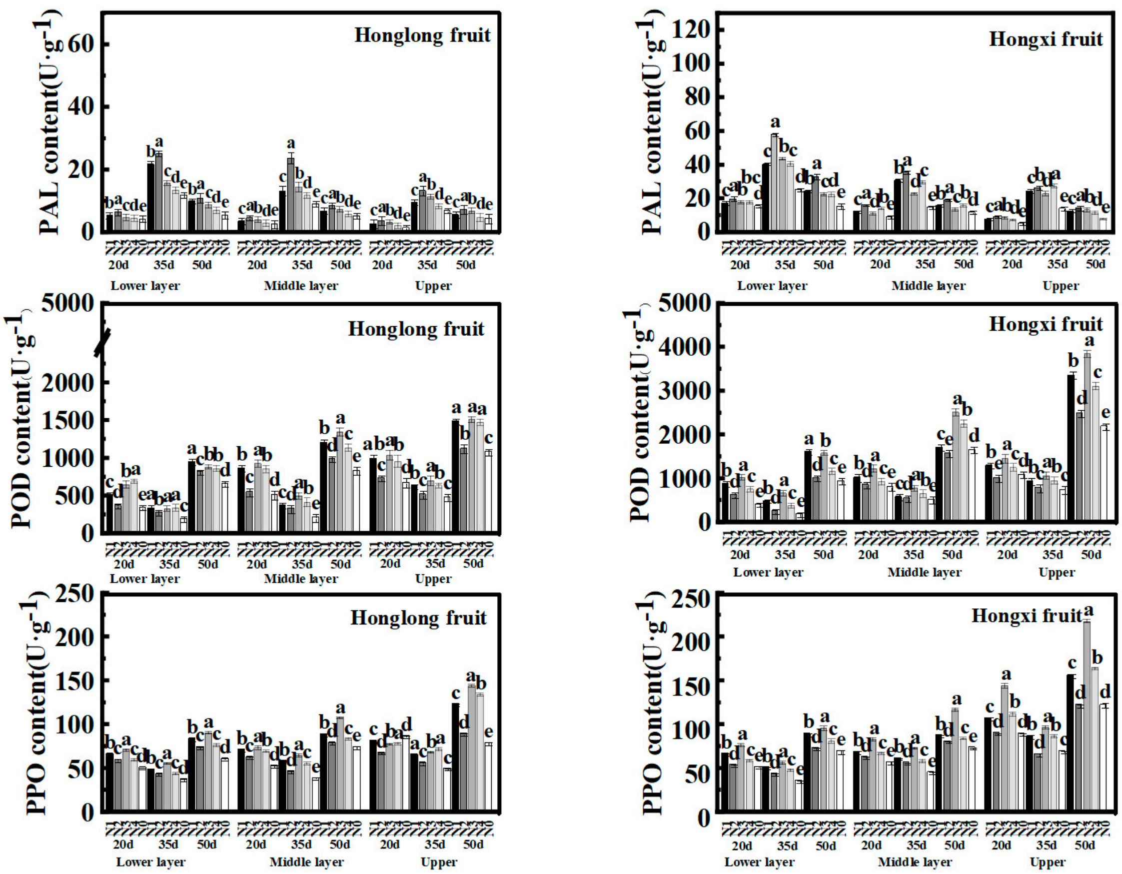

Figure 6. The effect of nitrogen fertilizer on the activities of phenylalanine ammonia-lyase (PAL), peroxidase (POD), and polyphenol oxidase(PPO) of dried chili pepper fruits. The data are presented as the mean ± standard error. Different lowercase letters indicate a significant difference among treatments according to the Duncan test ($p < 0.05$).

As illustrated in Figure 7, capsaicin content was positively correlated with PAL enzyme activity; the coefficient of capsaicin content with PAL enzyme activity was 0.85 for 'Honglong 23' and 0.79 for 'Hongxi'. Capsaicin content was negatively correlated with POD and PPO enzyme activities. The coefficient of POD enzyme activity was −0.16 for 'Honglong 23' and −0.44 for 'Hongxi'. The coefficient of PPO enzyme activity and capsaicin content was −0.45 for 'Honglong 23' and −0.48 for 'Hongxi'.

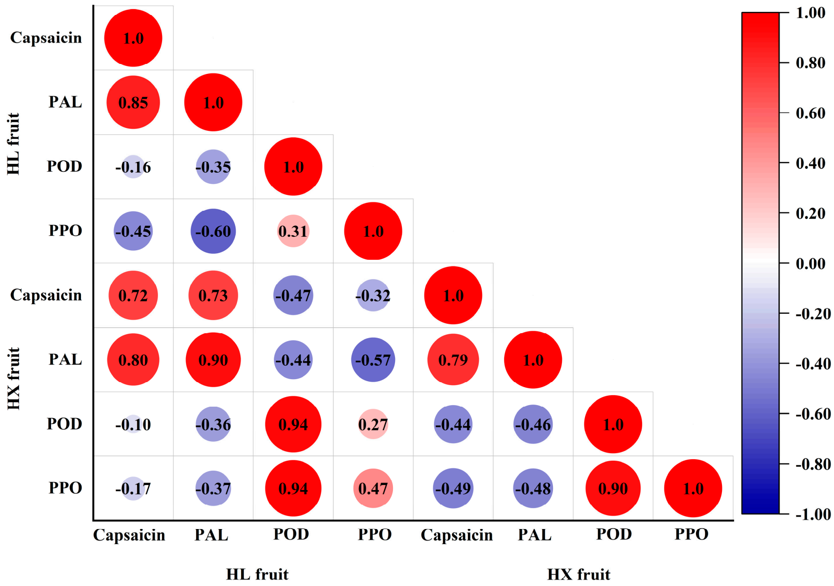

Figure 7. Correlation coefficients between capsaicinoid-related enzyme activities and capsaicinoid content of dried chili pepper. Note: The absolute value of a correlation coefficient higher than 0.30 is significant($p < 0.05$), and one higher than 0.80 is extremely significant ($p < 0.01$).

3.6. Effect of Nitrogen Fertilizer on the Expression of Capsaicin Synthetic Genes of the Dried Chili Pepper Fruit

As shown in Figure 8, the expression levels of capsaicinoid synthetic genes such as *PAL*, *AT3*, *C4H*, *4CL*, *COMT*, *HCT*, and *PAMT* were higher in the 'Hongxi' fruits than those in 'Honglong 23' and the expression levels of capsaicin synthetic genes of the two varieties ranked as 35th day > 50th day > 20th day. The expression levels of the seven capsaicin synthetic genes in different layers of the two varieties showed a tendency of bottom > middle > top, which is consistent with capsaicin content and capsaicin-related enzyme activity. Compared with N0, nitrogen application significantly upregulated the expression levels of capsaicin synthetic genes, and N2 showed the highest expression level of capsaicin synthetic genes. For example, the expression levels of AT3 with N2 of 'Honglong 23' and 'Hongxi' were a respective 1.48 and 2.32 times higher than that of N1 at the 35th day. This indicates that, within a certain range, the expression levels of capsaicin synthetic genes are positively related to the amount of nitrogen fertilizer. Overall, there were greater changes in capsaicin genes to nitrogen concentration of 'Hongxi' than that of 'Honglong 23', which indicates that 'Hongxi' is more sensitive to nitrogen.

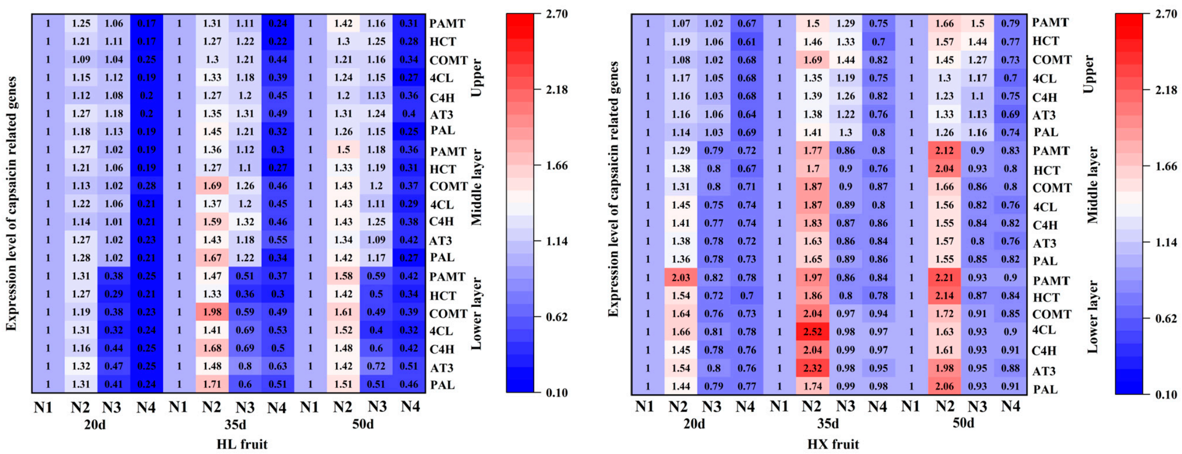

Figure 8. The effect of nitrogen fertilizer on the expression levels of capsaicin synthetic genes of dried chili pepper fruit. The data are presented as the mean ± standard error.

4. Discussion

4.1. Effect of Nitrogen Fertilizer on the Development of Pepper Fruits

Nitrogen is a key nutrient for plant growth and development. Nitrogen application can change the distribution and accumulation of nitrogen compounds in different plant organs. Studies of cotton have shown that reduction in nitrogen fertilizer properly increased the assimilate allocation in the fruit and then increased the fruit production [33]. It has been reported that, with the increase in nitrogen amount, the fruit weight of eggplant first increases and then decreases [34]. Studies on cantaloupe melons have shown that nitrogen application favors the fruit weight, length, diameter, and shape index [35]. Studies on tomatoes have shown that nitrogen application at 0.2 g/plant had a better promoting effect on fruit number, fruit dry weight, soluble sugar, Vc, and soluble solids content than 0.05 g/plant [36]. The above studies are in agreement with our study's performance. Studies on tomatoes have indicated that, compared with N0, the average fruit number was increased by 13.94%, 10.38%, and 10.68%, and yield was increased by 13.63%, 10.66%, and 8.42% in the N1 (150 kg ha^{-1}), N2 (300 kg ha^{-1}), and N3 (450 kg ha^{-1}) treatments, respectively. In this study, the fruit growth parameters treated with nitrogen were improved to varying degrees [37]. However, in this study, the growth index of chili pepper fruits gradually decreased as fruit position increased from bottom to top. There are two reasons for this: first, the nitrogen supply exceeded the pepper requirement, which broke the enzyme system and inhibited fruit growth [38]. The second reason is that excessive nitrogen fertilizer caused fruit abscission and reduced the fruit number [39]. Wu [40] found that, with the increase in fruit layers, the fruit weight of pepper gradually decreased, which is consistent with the results of this study. This may be related to the way in which fruits in higher layers were found to be smaller and less competitive for photosynthetic products [41].

4.2. Effect of Nitrogen Fertilizer on the Activity of Capsaicin Enzymes

Wang [42] has reported that, with an increase in urea amount, the peak time of capsaicinoids in pepper fruits is delayed, but that the peak area is increased. Medina et al. [43] have demonstrated that applying nitrogen increases the capsaicin content of fruits compared with N0. Han et al. [44] have demonstrated that capsaicin and dihydrocapsaicin exhibit first an increase first and then a decrease as the amount of nitrogen increases. Wang et al. [45] have found that 60% of the nitrogen amount favored the formation of capsaicinoids and dihydrocapsaicinoids, which is consistent with the higher capsaicinoid content of N2 in this study. The studies mentioned above are consistent with the trend, demonstrated in the present study, that capsaicin content is lower than N1 and gradually decreases from N2 to N4. However, Huang et al. [46] found a linear correlation between nitrogen amount and capsaicinoid content. The inconsistency with this study may be because capsaicin is a nitrogen compound, and its content is easily affected by nitrogen fertilizer; when nitrogen levels rise, capsaicin content increases. There was also a difference with the study of Lv et al. [47], in which capsaicin content was found to gradually decrease with an increase in nitrogen application. This is probably because nitrogen contributes to the vegetative growth of chili peppers. With the increase in nitrogen fertilizer dosage, the allocation of nitrogen to vegetative organs increases, further weakening the reproductive capacity of chili peppers, which leads to a decrease in the capsaicin synthesis of fruit. The significantly higher contents of capsaicinoids and dihydrocapsaicinoids in the placenta are because capsaicinoid synthesis is controlled by rate-limiting enzymes, and these enzymes are found first on the vesicular membrane of the epidermal cells of the placenta. Hence, the placenta is the starting site of capsaicin synthesis, and capsaicin in the fruit pulp comes from the transportation of the placenta [48]. Wang et al. [49] have claimed that capsaicin content reaches a maximum value at 28 days after anthesis and gradually decreases from 35 days after anthesis, which is inconsistent with this study. Wu's [50] study concluded that capsaicin content reaches its maximum value at the red ripening stage, which is inconsistent with our study. The differences in capsaicinoid content at different developmental stages may be due to the characteristics of different chili pepper varieties, such as the degradation enzyme activity and photo-oxidative decomposition of capsaicinoids. Bosland et al. [51] have found that the fruits in the second layer of pepper were the hottest, and that the spiciness decreases as the fruit position moves upward, which is in agreement with this study. Chen et al. [52] have found that the spiciness of pepper increases as the fruit position moves upward, which differs from the conclusion of this study. This could be related to the pepper varieties, nitrogen concentrations, and fertilizer application time. The decreased spiciness with the upward position of the fruit may be closely associated with factors such as fruit number and the relatively low competition for assimilates of the top layers of fruits. During intensive nutrition and reproductive growth, competition for assimilates between stems, leaves, and fruits increases, causing a gradual decrease in the capsaicinoid content of fruits [53].

POD is involved in the degradation of capsaicin. PPO mainly oxidizes phenolics, and, because capsaicin is a type of phenol, PPO may also be involved in its oxidative degradation [54]. Chen et al. [55] have reported that capsaicin content is positively correlated with PAL enzyme activity, negatively correlated with POD activity, and insignificantly correlated with PPO enzyme activity. Yang et al. [56] have demonstrated that PAL enzyme activity is positively correlated with capsaicin content, while POD is negatively correlated with capsaicin. In this study, the capsaicin content of 'Honglong 23' fruits was significantly lower than those of 'Hongxi', possibly due to differences in capsaicin synthesis and degradation enzyme activities. Furthermore, PAL enzyme activity was lower in 'Honglong 23' fruits and higher in 'Hongxi' fruits. PAL enzyme activity was lower than in the bottom layer fruits, and POD enzyme activity was higher in the top layer fruits. This results in higher capsaicinoid content of the bottom layer fruits than that of the top layers.

4.3. Effect of Nitrogen Fertilization on Capsaicin Precursors and Competitive Substances

Phenolic compounds are the precursors of capsaicin. Hall et al. [57] have indicated that there are different kinds of phenolic precursors, such as phenylalanine, p-coumaric acid, caffeic acid, and ferulic acid; these are also precursors of tannins, flavonoids, and lignans. Consequently, there is competition between the synthesis of capsaicinoids and tannins, flavonoids, and lignans. Ionică et al. [58] have demonstrated that the total phenolic and flavonoid contents increase with the development of chili pepper fruits, which is in contrast with this study. In our study, capsaicin content was found to be higher on the 35th day than on the 50th day, a difference that might be due to different varieties and sampling time. Chen et al. [59] have discovered that high capsaicin content is found on the 40th day after anthesis; at the same time, the contents of lignin and flavonoid are low, and when lignin and flavonoid content are high, capsaicin content should be reduced accordingly. This opposite variation in capsaicin content and competitors is consistent with the present study. In this study, more intermediate metabolites were allocated to synthesizing flavonoids, lignin, and tannin in fruits at the 50th day, which decreased capsaicin content. Furthermore, the total phenol content of 'Hongxi' was higher than that of 'Honglong 23', implying that there are more precursors for synthesizing capsaicinoids in 'Hongxi', thus contributing to its higher spiciness. Additionally, as the fruit position increases, the total phenol content decreases, as does the precursor substance, which in turn causes a lower capsaicin content in the top layers of fruits. It has been demonstrated that, when nitrogen fertilizer is deficient, the accumulation of nitrogen-free secondary metabolites such as terpenes and phenols increase, but when there is sufficient nitrogen fertilizer, the synthesis of nitrogen secondary metabolites increases [60].

4.4. Effect of Nitrogen Fertilizer on Capsaicin-Related Genes

Previous studies have found that the expressions of capsaicin synthetic genes such as PAL, AT3, 4CL, C4H, COMT, PAMT, and HCT are higher in the placenta than in the fruit pulp [61]. Li [62] found that the PAL, AT3, 4CL, and HCT expressions initially increase and then decrease during fruit development, which agrees with our results. Additionally, the expressions of *COMT* and *PAMT* continuously decrease. The expression of capsaicin synthetic genes varies among different varieties. Sarpras et al. [63] have stated that the expression of capsaicin synthetic genes, such as *PAL*, *C4H*, *COMT*, *AMT*, and *4CL,* differ with pepper varieties' spiciness. Generally, the expression of these genes is higher in spicy varieties, which is consistent with our finding that 'Hongxi' had a higher expression of capsaicin-synthetic genes than 'Honglong 23'.

4.5. Effect of Environmental Factors on Capsaicin

Besides fertilizer, there are many other factors to influence capsaicin synthesis. It has been reported that the highest capsaicin content in fruits is found at 80% soil moisture content, and that the lowest capsaicin content is found at 20% soil moisture content [64]. A study of different NaCl concentrations has revealed that 30 and 60 mM NaCl treatments increase capsaicin content compared with a control [65]. For temperature, some studies have supported the idea that capsaicin content decreases faster with the increase in temperature [66].

5. Conclusions

Amounts of 562.5 and 375 kg ha^{-1} of urea lead to the maximum values of pepper fruit weight, fruit diameter, fruit length, and placenta weight, so these are beneficial nitrogen concentrations for chili pepper yield and quality. Capsaicin accumulation exhibits a pattern of 35th day > 50th day> 20th day and bottom layers > middle layers > top layers of dried chili pepper. Competitive substances, such as lignin, flavonoids, tannins, and the degradative enzymes POD and PPO, increase with the increase in fruit position, while total phenol, PAL enzyme activity, and capsaicin synthetic gene expression decrease with fruit position. There were peaks in the phenol content, PAL enzyme activity, expression level of capsaicinoid synthetic genes, and valleys associated with competitive substances of N2 treatment.

Given capsaicin content, precursor substances, competitive substances, metabolic enzymes, and other capsaicin-related indicators, it was inferred that the most suitable nitrogen concentration was N2 (562.5 kg ha^{-1}). This study only collected one year of data, and the experiment needs more data from different regions over many years.

Reducing fertilizer pollution is an important direction for modern ecological agriculture. It is very common for excessive nitrogen to be applied to pepper. This experiment aimed to elucidate the effect of nitrogen fertilizer on capsaicin and determine the most conducive nitrogen amount for capsaicin accumulation. This will guide fertilization for pepper cultivation and contribute to the sustainable development of dried chili peppers.

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