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Responses of Growth, Enzyme Activity, and Flower Bud Differentiation of Pepper Seedlings to Nitrogen Concentration at Different Growth Stages

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Abstract: The concentration of nitrogen fertilizer is matched with the nutrient requirements in different growth stages of plants, which coordinates their vegetative and reproductive growth. In this study, the influences of nitrogen concentration before and after initiation of flower bud differentiation (first and second stage, respectively) on pepper seedling quality were studied. The chlorophyll a content, sucrose synthase activity, and sucrose phosphate synthase activity of pepper seedlings grown under moderate nitrogen (15 mmol L^{-1}) in the first stage combined with high nitrogen (25.61 mmol L^{-1}) in the second stage were 15.7%, 39.3%, and 34.6% higher than those of the same nitrogen concentration (15 mmol L^{-1}) in the first and second stages treatment, respectively. The regression model also showed that the values of flower bud diameter, shoot fresh weight, root fresh weight, and glutamine synthetase activity of pepper were high under the condition of moderate nitrogen in the first stage and higher nitrogen in the second stage. In addition, the results of comprehensive evaluation showed that moderate nitrogen (15 mmol L^{-1}) in the first stage and high nitrogen (25.61 mmol L^{-1}) in the second stage treatment ranked first, which improved carbon and nitrogen metabolism, increased biomass accumulation, and promoted the flower bud differentiation and flowering of pepper seedlings.

Keywords: chlorophyll content; comprehensive evaluation; flower bud; nitrogen concentration; pepper seedling

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1. Introduction

Pepper (*Capsicum annuum* L.) is one of the important economic and horticultural vegetables in the world, as its fruits are abundant in nutrients such as vitamin C, dietary fiber, and other compounds that are beneficial to human health [1–3]. Additionally, pepper is one of the primary vegetables cultivated year-round in protected horticulture, and the global annual production of pepper is estimated to be approximately 42.3 million tons, with a cultivation area of 3.7 million hectares [4]. Therefore, cultivating high-quality pepper seedlings is a vital way to increase crop yield and economic benefits [5,6].

In the growth of pepper plants, nitrogen is a crucial element involved in growth and development, serving as a fundamental component of proteins, nucleic acids, chlorophyll, and other essential organic molecules [7]. Insufficient nitrogen restricts plant growth and ultimately reduces vegetable yield. For instance, Flores-Saavedra et al. [8] indicated that tomato cultivated under a low nitrogen fertilization condition (0.5 mM) showed retarded development and reduced carbohydrates accumulation compared with those under high nitrogen treatment (5 mM); in addition, the photosynthesis and plant growth of tomato were enhanced with the increase in nitrogen content [9]. The application of appropriate nitrogen fertilizer significantly enhanced the uptake of phosphorus and potassium of plants and thereby regulated the growth and development of pepper and ultimately increased pepper yield [10,11]. Optimal nitrogen management enhances plant yield and resource use efficiency; conversely, excessive or insufficient nitrogen application can diminish plant yield, as well as reduce water use efficiency and nitrogen use efficiency [12]. In addition, excessive application of nitrogen also leads to environmental pollution [13]. Therefore, suitable nitrogen fertilizer management is a necessary method for annual production of pepper seedlings.

Flower bud differentiation is a key link during the growth and development of pepper, which is a sign of the transition from vegetative growth to reproductive growth [14]. Fertilization exerted a significant impact on flower bud differentiation, where the optimal ratios and application amounts of nitrogen, phosphorus, and potassium fertilizers were found to enhance nutrient absorption and promote flower bud differentiation in Chinese prickly ash [15]. Nitrogen plays an important role in flower bud differentiation, and previous studies have shown that nitrogen deficiency in the early growth stage of strawberry delayed flower bud differentiation, while optimal nitrogen concentration (0.1 g N per plug) at the seedling stage promoted flower bud differentiation [16,17]. Similarly, low nitrogen application inhibited crop growth, delayed leaf development, and reduced flowering, whereas the leaf growth and flowering of plants were promoted with increased nitrogen concentration [18]. However, excessive nitrogen application inhibited the flower formation of plants and reduced fruit yield [17]. Therefore, the optimization of a fertilization strategy could change the nutrient levels and regulate the flower bud differentiation and flowering of vegetable seedlings and ultimately affect crop yield and quality.

In the meantime, previous studies have shown that in the early stage of tomato fruit growth, excessive nitrogen reduced the water use efficiency of the plant [9]. In the middle or late stage of fruit growth, high nitrogen application increased the transpiration and water use efficiency [9]. The application of a high nitrogen concentration after flower budding increased the number of flower buds and flower fresh weight [19]. In addition, the precise adjustment of nutrient delivery in the key stages of pepper growth can regulate the growth and development of pepper [20,21]; flower bud differentiation and flowering were affected with the changes in metabolite content of plants [22]. However, most studies on the effects of nitrogen fertilizer on pepper growth have focused on nitrogen concentration or nitrogen form, and few studies investigated the influences of phased fertilization at different growth stages of pepper seedlings on flower bud differentiation and plant growth. In production, large amounts of nitrogen fertilizer uniformly from the beginning to the end of the seedling period were often applied due to the fact that farmers believe that increasing nitrogen fertilizer can increase plant yield, which not only causes environmental pollution but also increases production costs [23]. Therefore, the amount of fertilization applied at different growth stages should be coordinated with the nutrient requirements of plants at different growth stages, which can optimize biomass accumulation during both vegetative and reproductive growth stages, thereby influencing overall plant growth and development [24,25].

Therefore, our study aims to investigate the effects of nitrogen concentration at different growth stages on the biomass accumulation, photosynthetic pigment content, and carbon and nitrogen metabolic enzyme activity of pepper seedlings. Additionally, the effects of nitrogen concentration on flower bud differentiation and flowering of pepper were further investigated for optimizing nitrogen application. In addition, a comprehensive evaluation model and regression model were applied to determine the nitrogen fertilizer supply strategy at different growth stages of pepper seedlings. Our study could provide a feasible fertilization strategy to coordinate the growth and development of pepper seedlings and cultivate high-quality seedlings through the precise application of nitrogen fertilizer at different stages.

2. Materials and Methods

2.1. Plant Materials

Pepper (*Capsicum annuum* L. cv. Malayu) seeds were sown in 72-cell plug trays, using a substrate with a 3:1:1 volume ratio of peat, vermiculite, and perlite. The plug trays were kept in a closed transplant production system of Qingdao Agricultural University, Qingdao, Shandong Province, China. The light intensity and photoperiod were 250 μmol m^{-2} s^{-1} and 16 h d^{-1}, respectively, provided by white LEDs. The air temperature inside the closed transplant production system was 25 ± 2 °C for the light period and 18 ± 2 °C for the dark period, with a relative humidity of 60–70%.

2.2. Experimental Design

The nitrogen application level of the pepper seedlings at different growth stages was divided into the nitrogen concentration before initiation of flower bud differentiation (the first stage) and the nitrogen concentration after initiation of flower bud differentiation (the second stage). The initiation of flower bud differentiation was determined when the growth cone of the pepper seedling widened and a round protrusion appeared, according to the method reported by Liu et al. [26]. A central composite design of two factors and five levels with the codes (−1.414, −1, 0, 1, 1.414) were adopted in the experiment [27], and the corresponding nitrogen concentration was 4.39, 7.5, 15, 22.5, and 25.61 mmol L^{-1}, respectively (Table 1). This experiment was conducted based on the principle of quadratic regression orthogonal design (Table 2), which can arrange more experimental gradients with fewer treatments and is widely used in biological experiments [28]. Therefore, this experiment consisted of nine treatments (T5 was considered as the control) with three replicates per treatment and 72 pepper seedlings per replicate (Table 3).

Table 1. Experimental design of nitrogen concentration applied for pepper seedlings at different growth stages.

Growth Stages	Nitrogen Concentration Code and Level (mmol L^{-1})				
	−1.414	**−1**	**0**	**1**	**1.414**
Before initiation of flower bud differentiation	4.39	7.5	15	22.5	25.61
After initiation of flower bud differentiation	4.39	7.5	15	22.5	25.61

Codes (−1.414, −1, 0, 1, 1.414) represent the level at different treatment factors.

Table 2. Two-factor experiment design based on the principle of the quadratic regression orthogonal design.

Nitrogen Concentration before Initiation of Flower Bud Differentiation (mmol L^{-1})	Nitrogen Concentration after Initiation of Flower Bud Differentiation (mmol L^{-1})				
	4.39	**7.5**	**15**	**22.5**	**25.61**
4.39			1		
7.5		1		1	
15	1		1		1
22.5		1		1	
25.61			1		

"1" represents the combination of experimental treatments performed.

Table 3. Treatments of nitrogen concentration at different growth stages of pepper seedlings.

Treatment	Nitrogen Concentration before Initiation of Flower Bud Differentiation (mmol L^{-1})	Nitrogen Concentration after Initiation of Flower Bud Differentiation (mmol L^{-1})
T1	4.39	15
T2	7.5	7.5

Table 3. *Cont.*

Treatment	Nitrogen Concentration before Initiation of Flower Bud Differentiation (mmol L^{-1})	Nitrogen Concentration after Initiation of Flower Bud Differentiation (mmol L^{-1})
T3	7.5	22.5
T4	15	4.39
T5	15	15
T6	15	25.61
T7	22.5	7.5
T8	22.5	22.5
T9	25.61	15

Nitrogen concentration before and after initiation of flower bud differentiation were experimental variables. There were 9 treatments in this experiment, and T5 was the control.

In this study, Hoagland solution with nitrogen concentration of 15 mmol L^{-1} was used, which contained (mg L^{-1}) $Ca(NO_3)_2 \cdot 4H_2O$ (945), KNO_3 (607), $NH_4H_2PO_4$ (115), and $MgSO_4 \cdot 7H_2O$ (493) and the micronutrients Na_2Fe-EDTA (30), H_3BO_3 (2.86), $MnSO_4 \cdot 4H_2O$ (2.13), $ZnSO_4 \cdot 7H_2O$ (0.22), $CuSO_4 \cdot 5H_2O$ (0.08), and $(NH_4)_6Mo_6O_{24} \cdot 4H_2O$ (0.02). The low level of nitrogen concentrations (4.39 mmol L^{-1} and 7.5 mmol L^{-1}) were prepared by decreasing the contents of $Ca(NO_3)_2$ and KNO_3 in the solution and increasing the contents of $CaCl_2$ and KCl in the above solution. The high level of nitrogen concentrations (22.5 mmol L^{-1} and 25.61 mmol L^{-1}) were prepared by adding $NaNO_3$ to the solution. The pH values were maintained between 6.0 and 6.5, and the pepper seedlings were irrigated with 1/2 concentration nutrient solution at the cotyledon stage, and full concentration nutrient solution was applied every 3 days after the first true leaf appeared. The destructive measurements of the pepper seedlings were carried out at 40 days after sowing. Subsequently, the pepper seedlings were transplanted into plastic pots (diameter, 8.5 cm; depth, 7 cm) with mixed peat, vermiculite, and perlite (3:1:1, *v*/*v*/*v*), which were sampled for flower observation at 60 days after sowing.

2.3. Growth Measurement

2.3.1. Plant Growth Parameters

Plant growth parameters of pepper seedlings were measured at 40 days after sowing. Root surface area and root volume of pepper seedlings were measured using WinRHIZO software (Version 2016a, Regent Instruments Inc., Quebec, QC, Canada). Shoot fresh weight and root fresh weight of pepper seedlings were determined with an electronic analytical balance (FA1204B; BioonGroup, Shanghai, China). Fresh shoots and roots of pepper seedlings were dried in an oven at 105 °C for 3 h, and the dry weights of the shoots and roots were determined after further drying at 80 °C for 72 h. The seedling quality index was determined based on Yan et al. [29].

2.3.2. Photosynthetic Pigment Content

Photosynthetic pigment content of pepper leaves was measured at 40 days after sowing. About 0.2 g of mature pepper leaves were cut into pieces and soaked in 10 mL of 95% alcohol for 48 h. Absorbance of the extract at wavelength 665 nm, 649 nm and 470 nm was measured by a spectrophotometer (1810, Shanghai Yoke Instrument Co., Ltd., Shanghai, China) according to Xing et al. [30].

2.3.3. Enzyme Activity

Enzyme activity of pepper leaves was measured at 40 days after sowing. The nitrate reductase (NR) activity and glutamine synthetase (GS) activity was determined by the method of NR Kit (BC0080, Solarbio, Beijing, China) [31] and GS Kit (BC0910, Solarbio, Beijing, China) [32], respectively. Sucrose synthase (SS) activity and sucrose phosphate synthase (SPS) activity was determined using the SS Kit (BC0580, Solarbio, Beijing, China) and SPS Kit (BC0600, Solarbio, Beijing, China), respectively, following the instruction manual [33].

2.3.4. Flower Buds of Pepper Seedlings

The flower bud diameter of the pepper seedlings was measured at 40 days after sowing with a vernier caliper, dissected with anatomic needle, and photographed under a microscope (SMZ-140, Motic, Wetzlar, Germany).

2.3.5. Flower Organs of Pepper Seedlings

Flower organs of pepper seedlings were measured at 60 days after sowing, photographed under a microscope (SMZ-140, Motic, Wetzlar, Germany), dissected with anatomic needle, and flower transverse diameter, flower longitudinal diameter, anther length, anther width, filament length, stigma width, and style length were measured with a vernier caliper.

2.3.6. Comprehensive Evaluation Methods

An entropy weighted technique for order preference by similarity to an ideal solution (TOPSIS) method was used to determine the weight of each index, and the comprehensive evaluation scores of treatments were ranked based on previous studies [34,35].

2.3.7. Regression Model

Multiple regression fitting of the data was performed through using Origin 2021 to determine the model coefficients, and then the growth model was generated using the equations. The relationship model was established through a binary quadratic equation, as shown in Equation (1).

$$z = a + bx + cy + dx^2 + ey^2 + fxy \quad (1)$$

where x and y indicate the input, z indicates the output. The six items on the right side of the equation represent constant terms, linear terms, quadratic terms, and interaction terms.

2.4. Date Analysis

All data were analyzed for significance by one-way ANOVA using DPS 7.05 (Hangzhou Ruifeng Information Technology Co., Ltd., Hangzhou, China), and the significance differences among treatments was conducted by the Least Significant Difference (LSD) method at the $p < 0.05$ level. Origin 2021 (Origin Lab, Northampton, MA, USA) was used for correlation analysis.

3. Results

3.1. Effects of Nitrogen Concentration at Different Growth Stages on Biomass and Seedling Quality Index of Pepper Seedlings

Nitrogen concentration at different growth stages had significant influences on the shoot and root weight of the pepper seedlings (Table 4). The shoot fresh weight, shoot dry weight, and root fresh weight of pepper seedlings exposed to the T5 treatment increased by 41.9%, 33.3%, and 16.0% compared with those under the T1 treatment, respectively. The shoot fresh weight, shoot dry weight, and root fresh weight of pepper seedlings exposed to the T5 treatment increased by 20.1%, 23.1%, and 17.0% compared with those under the T9 treatment, respectively. The seedling quality index of pepper seedlings exposed to the T5 treatment was increased by 36.4% and 31.8% compared with those in the T1 and T9 treatments, respectively. The shoot fresh weight, shoot dry weight, and seedling quality index of pepper seedlings grown under the T5 treatment were increased by 22.2%, 12.8%, and 13.6% compared with those under T4 treatment, respectively. However, there were no remarkable disparities in the biomass and seedling quality index of pepper seedlings between the T5 and T6 treatments.

Table 4. Effects of nitrogen concentration at different growth stages on biomass and seedling quality index of pepper seedlings.

Treatment	Shoot Fresh Weight (g per Plant)	Root Fresh Weight (g per Plant)	Shoot Dry Weight (g per Plant)	Root Dry Weight (g per Plant)	Seedling Quality Index
T1	2.43 ± 0.06 c	0.79 ± 0.04 cd	0.26 ± 0.02 d	0.063 ± 0.002 abc	0.14 ± 0.01 c
T2	2.50 ± 0.02 c	0.83 ± 0.07 bcd	0.29 ± 0.02 d	0.065 ± 0.004 abc	0.14 ± 0.03 c
T3	3.19 ± 0.23 b	0.83 ± 0.03 bcd	0.35 ± 0.02 bc	0.063 ± 0.007 bc	0.15 ± 0.02 c
T4	3.25 ± 0.18 b	0.86 ± 0.07 abcd	0.34 ± 0.02 c	0.069 ± 0.007 abc	0.19 ± 0.02 b
T5	4.18 ± 0.17 a	0.94 ± 0.04 ab	0.39 ± 0.02 a	0.070 ± 0.006 abc	0.22 ± 0.01 a
T6	4.57 ± 0.52 a	0.98 ± 0.07 a	0.39 ± 0.04 ab	0.072 ± 0.005 ab	0.19 ± 0.01 ab
T7	3.32 ± 0.63 b	0.76 ± 0.19 d	0.36 ± 0.03 abc	0.062 ± 0.005 c	0.17 ± 0.02 bc
T8	4.44 ± 0.06 a	0.93 ± 0.1 abc	0.39 ± 0.01 a	0.072 ± 0.006 a	0.19 ± 0.02 ab
T9	3.34 ± 0.14 b	0.78 ± 0.06 cd	0.30 ± 0.03 d	0.064 ± 0.006 abc	0.15 ± 0.02 c

Data are mean ± standard deviation. Different lowercase letters indicated significant differences among treatments at the $p < 0.05$ level by Least Significant Difference (LSD) test.

3.2. Effects of Nitrogen Concentration at Different Growth Stages on Root Growth of Pepper Seedlings

The root surface area of pepper seedlings under the T6 treatment exhibited a maximum value, and root volume of pepper seedlings under T5 treatment exhibited a maximum value; however, there were no significant differences in the root surface area and root volume of pepper seedlings among the T4, T5, and T6 treatments (Figure 1). The root surface area and root volume of pepper seedlings exposed to the T5 treatment increased by 28.1% and 24.3% compared with those under the T1 treatment, respectively. The root surface area and root volume of pepper seedlings exposed to the T5 treatment improved by 30.5% and 25.0% contrasted with those in T9 treatment, respectively.

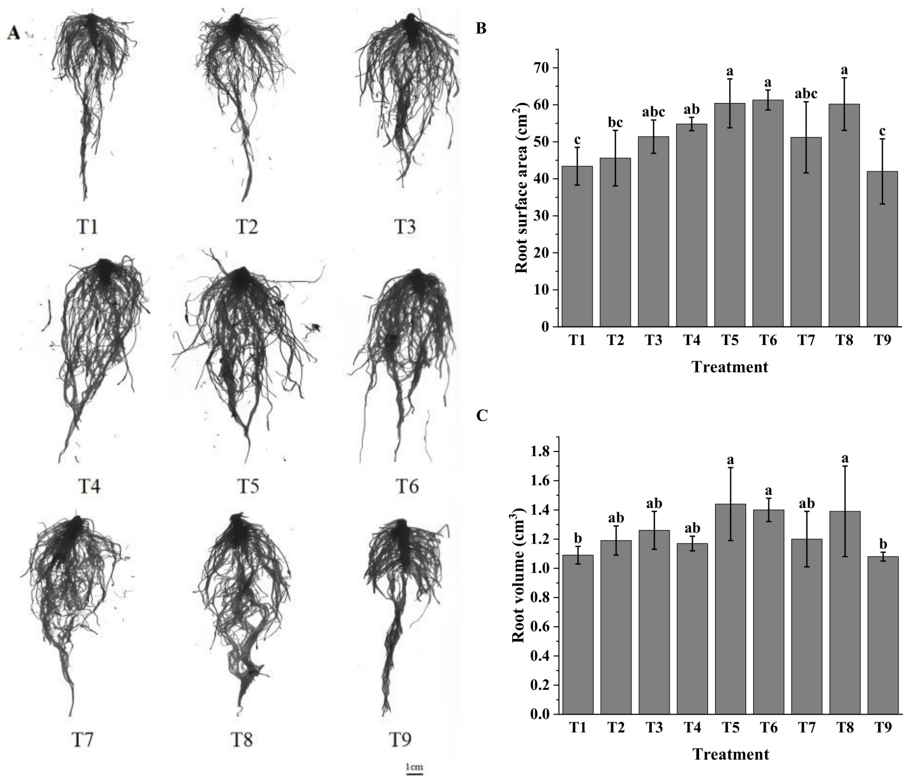

Figure 1. Effects of nitrogen concentration at different growth stages on root morphology of pepper seedlings. (**A**) Root morphology. (**B**) Root surface area. (**C**) Root volume. Error bars indicate the standard deviation. Different lowercase letters indicated significant differences among treatments at the $p < 0.05$ level by Least Significant Difference (LSD) test.

3.3. Effects of Nitrogen Concentration at Different Growth Stages on Photosynthetic Pigment Content of Pepper Seedlings

The chlorophyll a content, chlorophyll b content, carotenoid content, and total chlorophyll content of pepper seedlings cultivated under the T5 treatment were significantly increased by 19.3%, 35.2%, 20.0%, and 23.5% compared with those under the T4 treatment, respectively (Table 5). The chlorophyll a content and total chlorophyll content of pepper seedlings cultivated under the T6 treatment were significantly increased by 15.7% and 12.8% compared with those under the T5 treatment, respectively. However, there were no remarkable disparities in the chlorophyll b content and carotenoid content of pepper seedlings between the T5 and T6 treatments. The chlorophyll a content, chlorophyll b content, carotenoid content, and total chlorophyll content of pepper seedlings cultivated under the T5 treatment were significantly increased by 46.0%, 55.6%, 40.0%, and 48.5% compared with those under T1 treatment, respectively.

Table 5. Effects of nitrogen concentration at different growth stages on photosynthetic pigment content of pepper seedlings.

Treatment	Chlorophyll a Content (mg g^{-1})	Chlorophyll b Content (mg g^{-1})	Carotenoid Content (mg g^{-1})	Total Chlorophyll Content (mg g^{-1})
T1	0.81 ± 0.05 e	0.24 ± 0.03 e	0.21 ± 0.02 d	1.05 ± 0.08 e
T2	1.33 ± 0.01 cd	0.37 ± 0.01 d	0.31 ± 0.00 bc	1.70 ± 0.02 d
T3	1.54 ± 0.04 abc	0.45 ± 0.01 c	0.35 ± 0.01 ab	1.99 ± 0.04 c
T4	1.21 ± 0.24 d	0.35 ± 0.07 d	0.28 ± 0.06 c	1.56 ± 0.29 d
T5	1.50 ± 0.05 bc	0.54 ± 0.02 b	0.35 ± 0.01 ab	2.04 ± 0.07 bc
T6	1.78 ± 0.10 a	0.56 ± 0.04 b	0.38 ± 0.02 a	2.34 ± 0.13 a
T7	1.70 ± 0.07 ab	0.51 ± 0.01 b	0.37 ± 0.02 a	2.21 ± 0.09 abc
T8	1.75 ± 0.34 ab	0.64 ± 0.03 a	0.39 ± 0.07 a	2.39 ± 0.34 a
T9	1.74 ± 0.12 ab	0.56 ± 0.02 b	0.38 ± 0.03 a	2.31 ± 0.12 ab

Data are mean ± standard deviation. Different lowercase letters indicated significant differences among treatments at the $p < 0.05$ level by Least Significant Difference (LSD) test.

3.4. Effects of Nitrogen Concentration at Different Growth Stages on Enzyme Activity of Pepper Seedlings

The sucrose synthase activity of pepper seedlings in the T6 treatment was higher than those under the T4 and T5 treatments, which were 47.9% and 39.3% higher than those of the T4 and T5 treatments, respectively (Figure 2). Similarly, the sucrose phosphate synthase activity of pepper seedlings cultivated under the T6 treatment was increased by 58.6% and 34.6% compared with that under the T4 and T5 treatments, respectively. The sucrose phosphate synthase activity of pepper seedlings grown under the T5 treatment was significantly increased compared with those under the T1 and T9 treatments. The sucrose synthase activity, sucrose phosphate synthase activity, nitrate reductase activity, and glutamine synthetase activity of pepper seedlings in the T5 treatment were remarkably increased compared with those under the T1 treatment.

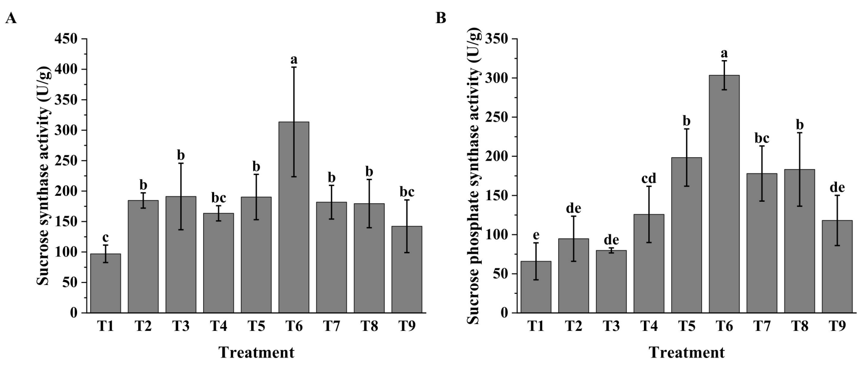

Figure 2. *Cont.*

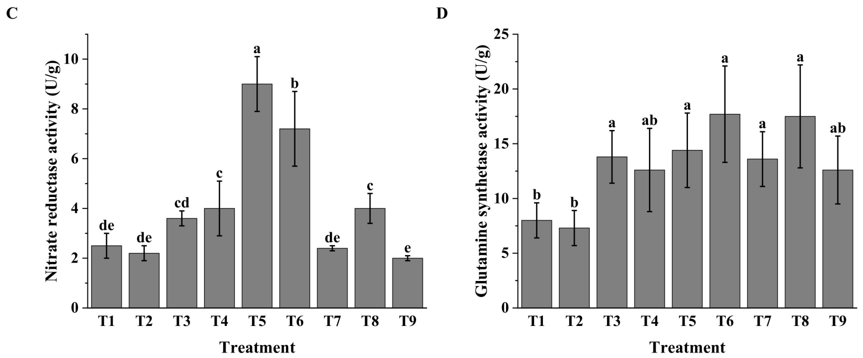

Figure 2. Effects of nitrogen concentration at different growth stages on enzyme activity of pepper seedlings. (**A**) Sucrose synthase activity. (**B**) Sucrose phosphate synthase activity. (**C**) Nitrate reductase activity. (**D**) Glutamine synthetase activity. Error bars indicate the standard deviation. Different lowercase letters indicated significant differences among treatments at the $p < 0.05$ level by Least Significant Difference (LSD) test.

3.5. Effects of Nitrogen Concentration at Different Growth Stages on Flower Bud Differentiation, Flowering and Flower Organ Development of Pepper

The maximum flower bud diameter of pepper seedlings occurred in the T6 treatment, which was increased by 20.4% compared with that in the T4 treatment (Figure 3 and Table 6). However, there was no significance in the flower bud diameter of pepper seedlings between the T5 and T6 treatments. The flower bud diameter of pepper seedlings exposed to the T5 treatment was increased by 30.6% and 22.5% contrasted with those in the T1 and T9 treatments, respectively.

The flowers of pepper seedlings cultivated under the T6 treatment were larger compared with those under the T4 and T5 treatments (Figure 3). The flowers of pepper seedlings cultivated under the T5 treatment were significantly larger compared with those under the T1 and T9 treatments. The flower transverse diameter and flower longitudinal diameter were the highest for pepper seedlings cultivated under the T6 treatment, whereas no significant differences were observed in the flower transverse diameter and flower longitudinal diameter of pepper seedlings cultivated between the T5 and T6 treatments (Table 6). The flower transverse diameter and flower longitudinal diameter of pepper seedlings cultivated under the T5 treatment increased by 67.0% and 66.3% compared with those under the T1 treatment. The anther length and anther width of pepper seedling flowers exposed to the T1 treatments increased by 26.2% and 37.0% compared with those under the T5 treatment, respectively.

Figure 3. Effects of nitrogen concentration at different growth stages on flower bud differentiation and flowering of pepper seedlings. (**A**) Flower bud anatomy (40 days after sowing). (**B**) Flower (60 days after sowing).

Table 6. Effects of nitrogen concentration at different growth stages on flower bud diameter and flower organ development of pepper seedlings.

Treatment	Flower Bud Diameter/mm	Flower Transverse Diameter /mm	Flower Longitudinal Diameter/mm	Anther Length/mm	Anther Width/mm	Filament Length/mm	Stigma Width/mm	Style Length/mm
T1	1.45 ± 0.20 c	6.3 ± 0.2 d	6.0 ± 0.4 c	2.63 ± 0.09 a	1.81 ± 0.16 a	1.75 ± 0.07 c	0.64 ± 0.06 bc	4.51 ± 0.45 c
T2	1.45 ± 0.35 c	10.0 ± 1.4 d	9.8 ± 1.2 b	2.40 ± 0.55 ab	1.33 ± 0.45 bc	3.00 ± 0.22 a	0.54 ± 0.02 c	5.8 ± 0.28 b
T3	1.84 ± 0.15 abc	19.6 ± 3.3 a	12.3 ± 2.4 b	2.06 ± 0.06 ab	1.50 ± 0.07 ab	2.48 ± 0.34 ab	0.73 ± 0.02 ab	6.51 ± 0.43 ab
T4	1.79 ± 0.12 bc	15.2 ± 3.7 bc	10.5 ± 2.8 b	1.91 ± 0.38 b	1.23 ± 0.11 bc	2.76 ± 0.30 ab	0.72 ± 0.04 ab	6.33 ± 0.20 ab
T5	2.09 ± 0.02 ab	19.1 ± 2.0 a	17.8 ± 2.2 a	1.94 ± 0.26 b	1.14 ± 0.23 bc	2.59 ± 0.83 ab	0.71 ± 0.12 ab	7.17 ± 0.52 a
T6	2.25 ± 0.60 a	20.7 ± 1.1 a	18.2 ± 4.0 a	2.06 ± 0.07 ab	1.16 ± 0.31 bc	2.71 ± 0.36 ab	0.81 ± 0.05 a	7.09 ± 0.50 a
T7	1.83 ± 0.19 abc	14.9 ± 3.8 c	12.3 ± 1.2 b	1.82 ± 0.39 b	1.12 ± 0.09 c	2.42 ± 0.02 abc	0.81 ± 0.04 a	7.07 ± 0.48 a
T8	2.19 ± 0.04 ab	19.7 ± 1.4 a	17.7 ± 2.1 a	2.05 ± 0.16 ab	1.24 ± 0.11 bc	2.89 ± 0.53 ab	0.72 ± 0.17 ab	7.12 ± 0.14 a
T9	1.62 ± 0.12 c	6.2 ± 0.1 d	5.9 ± 0.5 c	2.42 ± 0.65 ab	1.70 ± 0.04 a	2.17 ± 0.50 bc	0.65 ± 0.03 bc	5.55 ± 1.31 b

Flower buds were sampled 40 days after sowing, and flower organs were sampled 60 days after sowing. Data are mean ± standard deviation. Different lowercase letters indicated significant differences among treatments at the $p < 0.05$ level by Least Significant Difference (LSD) test.

3.6. Correlation Analysis between Morphological and Physiological Indicators of Pepper Seedlings

The correlation coefficients between the flower bud diameter of pepper seedlings and shoot fresh weight, root fresh weight, shoot dry weight, seedling quality index, sucrose phosphate synthase activity, and glutamine synthetase activity were 0.97, 0.83, 0.96, 0.82, 0.84, and 0.95, respectively, reaching a highly significant positive correlation level ($p < 0.01$) (Figure 4). The correlation coefficients between the flower bud diameter of pepper seedlings and root dry weight, chlorophyll b content, total chlorophyll content, sucrose synthase activity, and nitrate reductase activity were 0.77, 0.75, 0.68, 0.71, and 0.74, respectively, showing a significant positive correlation ($p < 0.05$). The correlation coefficients between the seedling quality index of pepper seedlings and shoot fresh weight, shoot dry weight, flower bud diameter, and nitrate reductase activity were 0.81, 0.84, 0.82, and 0.85, respectively, which reached a highly significant correlation level ($p < 0.01$).

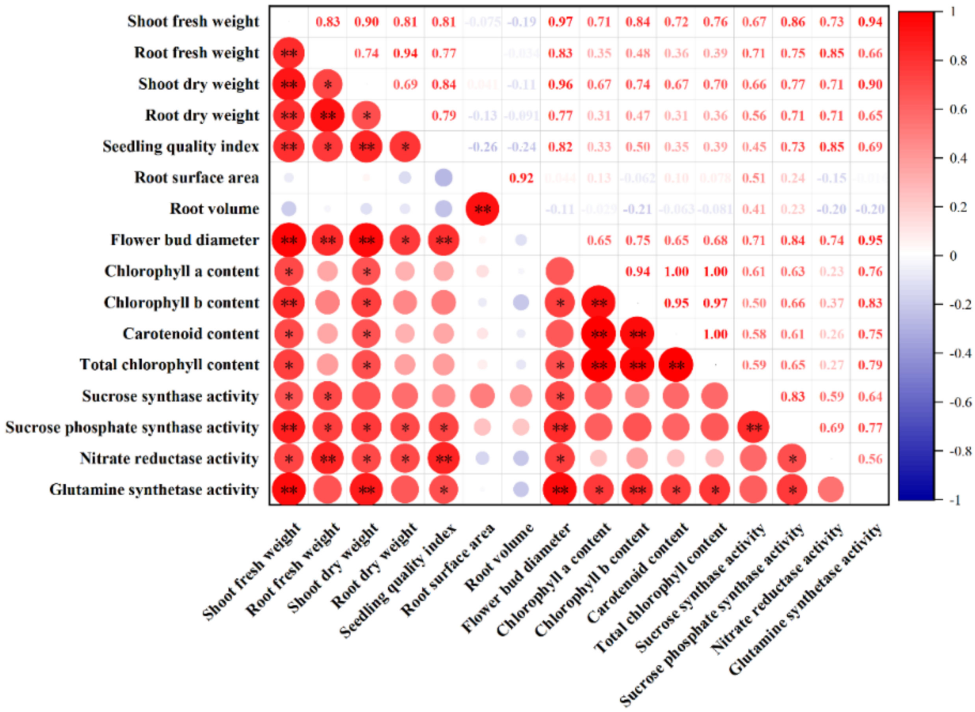

Figure 4. Correlation analysis between morphological and physiological indicators of pepper seedlings. * and ** indicated significance at $p < 0.05$ and 0.01, respectively.

3.7. Comprehensive Evaluation Based on Entropy Weight and TOPSIS

The comprehensive evaluation of nine treatments was evaluated by an entropy weight TOPSIS method. Firstly, the entropy weight method was used to determine the weight of each indicator; the shoot fresh weight, root fresh weight, root dry weight, seedling quality index, root surface area, root volume, flower bud diameter, sucrose phosphate synthase activity, and nitrate reduction activity parameters provided a greater weight for comprehensive evaluation (Table 7). Secondly, the ranking of the comprehensive evaluation of the nine treatments was determined by TOPSIS; the results showed that the T6 treatment ranked first, followed by the T5 and T8 treatments. However, the T1 and T9 treatments ranked ninth and eighth, respectively (Table 8).

Table 7. The weights of all indicators of pepper seedlings grown under nitrogen concentrations at different growth stages based on entropy.

Indicator	Information Entropy Value	Information Utility Value	Weight Coefficient
Shoot fresh weight	0.860	0.140	6.44%
Root fresh weight	0.835	0.165	7.60%
Shoot dry weight	0.903	0.097	4.47%
Root dry weight	0.824	0.176	8.12%
Seedling quality index	0.802	0.198	9.11%
Root surface area	0.863	0.137	6.32%
Root volume	0.841	0.159	7.31%
Flower bud diameter	0.840	0.160	7.37%
Chlorophyll a content	0.930	0.070	3.23%
Chlorophyll b content	0.912	0.088	4.06%
Carotenoid content	0.929	0.071	3.27%
Total chlorophyll content	0.927	0.073	3.37%
Sucrose synthase activity	0.900	0.100	4.62%
Sucrose phosphate synthase activity	0.831	0.169	7.76%
Nitrate reductase activity	0.740	0.260	11.97%
Glutamine synthetase activity	0.892	0.108	4.99%

Table 8. The comprehensive evaluation and sorting of pepper seedlings using TOPSIS for all the treatments.

Treatment	D_i^+	D_i^-	C_i	Comprehensive Evaluation Sorting
T1	0.244	0.050	0.170	9
T2	0.219	0.108	0.331	7
T3	0.194	0.099	0.338	6
T4	0.165	0.115	0.411	5
T5	0.108	0.213	0.663	2
T6	0.046	0.241	0.839	1
T7	0.186	0.133	0.417	4
T8	0.121	0.188	0.608	3
T9	0.225	0.082	0.267	8

D_i^+ and D_i^-, respectively, are the distance between the evaluation object and the positive and negative ideal solutions. C_i is the degree of proximity between the evaluation object and the optimal scheme.

3.8. Regression Models of Nitrogen Concentration at Different Growth Stages on Shoot Fresh Weight, Shoot Dry Weight, Flower Bud Diameter, and Glutamine Synthetase Activity of Pepper Seedlings

Shoot fresh weight, root fresh weight, and flower bud diameter showed similar changes under different nitrogen concentrations at different growth stages (Figure 5). The linear coefficient of the regression equation showed that nitrogen concentration before and after initiation of flower bud differentiation had positive effects on the shoot fresh weight, root fresh weight, and flower bud diameter of pepper seedlings, while the quadratic coefficient showed that excessive nitrogen concentration before and after initiation of flower bud differentiation had negative effects (Table 9). Similarly, the linear coefficient showed that nitrogen concentration before and after initiation of flower bud differentiation had a positive influence on the glutamine synthetase activity of pepper seedlings; the secondary coefficient showed that excessive nitrogen concentration before initiation of flower bud differentiation had an inhibitory effect on glutamine synthetase activity, but higher nitrogen concentration after initiation of flower bud differentiation had a positive effect. The three-dimensional model showed that moderate nitrogen concentration before initiation of flower bud differentiation and higher after initiation of flower bud differentiation could improve the shoot fresh weight, root fresh weight, flower bud diameter, and glutamine synthetase activity of pepper seedlings.

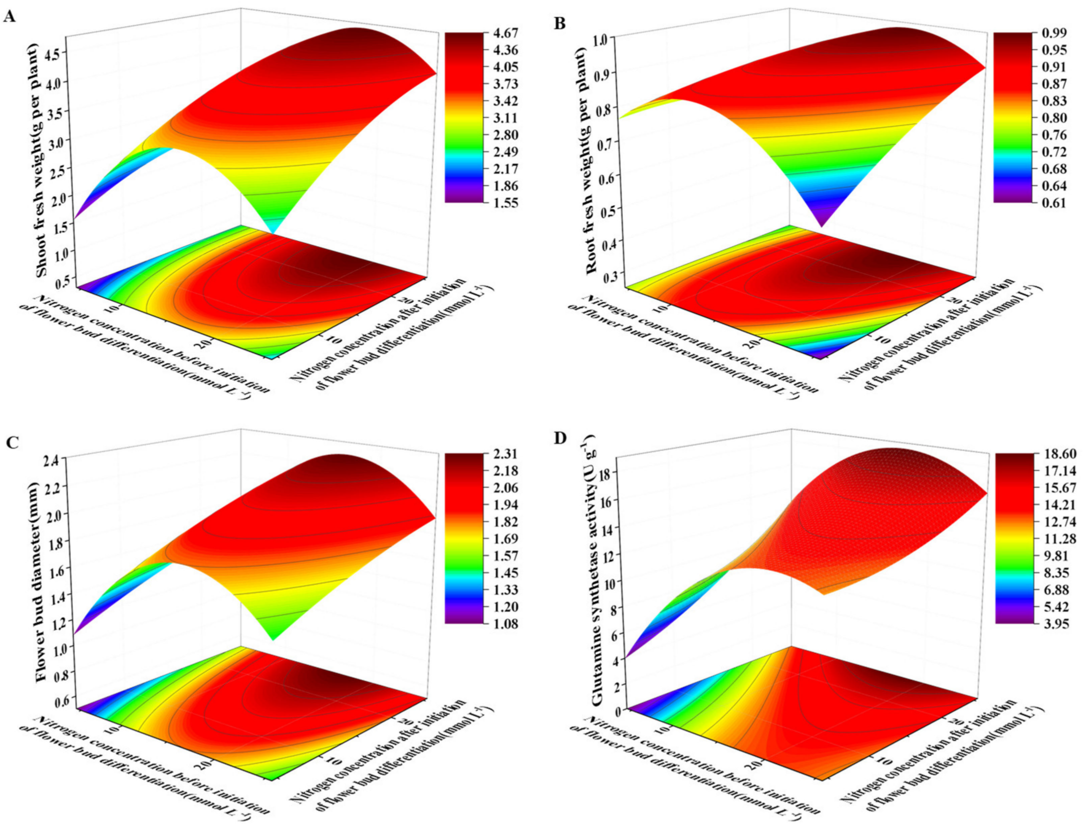

Figure 5. Regression models of nitrogen concentration at different growth stages on (**A**) shoot fresh weight, (**B**) root fresh weight, (**C**) flower bud diameter, and (**D**) glutamine synthetase activity of pepper seedlings. x and y axis indicated nitrogen concentration before initiation of flower bud differentiation (mmol L^{-1}) and nitrogen concentration after initiation of flower bud differentiation (mmol L^{-1}), respectively.

Table 9. Regression equation of nitrogen concentration at different growth stages on shoot fresh weight, root fresh weight, flower bud diameter, and glutamine synthetase activity of pepper seedlings.

Parameters	Equation	R^2	F-Value	*p*-Value
Shoot fresh weight	$z = -0.3405 + 0.37686x + 0.10856y - 0.01166x^2 - 0.00254y^2 + 0.00193xy$	0.96	37.43	0.007
Root fresh weight	$z = 0.62201 + 0.03349x + 0.0031y - 0.00147x^2 - 2.86578E - 4y^2 + 7.51111E - 4xy$	0.96	42.43	0.006
Flower bud diameter	$z = 0.31752 + 0.15946x + 0.03737y - 0.00471x^2 - 4.012E - 4y^2 - 1.33333E - 4xy$	0.86	11.14	0.037
Glutamine synthetase activity	$z = -2.89352 + 1.49733x + 0.23693y - 0.03472x^2 + 0.00792y^2 - 0.012xy$	0.92	20.41	0.016

x, y in the formula indicated nitrogen concentration before initiation of flower bud differentiation (mmol L^{-1}) and nitrogen concentration after initiation of flower bud differentiation (mmol L^{-1}), respectively.

4. Discussion

4.1. Effects of Nitrogen Concentration at Different Growth Stages on Growth of Pepper Seedlings

Plant growth and development can be affected by nitrogen fertilizer and other environmental conditions [10,36]. In this study, under the condition of the same nitrogen concentration (15 mmol L^{-1}) in the second stage, moderate nitrogen (15 mmol L^{-1}) in the first stage significantly promoted the shoot biomass, root fresh weight, seedling quality index, root surface area, and root volume of pepper seedlings compared with low or high nitrogen (4.39 and 25.61 mmol L^{-1}) (Table 4 and Figure 1). Concurring with our findings, a previous study showed that the shoot dry weight and leaf area of pepper plants were significantly reduced with the decrease in nitrogen fertilizer supply [10]. Similar results were found in tomato and lettuce, where the plant growth was delayed and dry weight of plants was lower under low nitrogen conditions compared with high nitrogen conditions [8,37]. However, excessive nitrogen application reduced the plants' yield and nitrogen use efficiency [12]. These results demonstrated that appropriate nitrogen concentration in the early growth stage of pepper seedlings increased the leaf area, the photosynthesis of leaves and thus promoted plant biomass and seedling quality, while insufficient or excessive nitrogen could lead to metabolic disorders in plants and inhibit pepper growth. Furthermore, under the condition of appropriate nitrogen concentration in the first stage, high nitrogen concentration in the second stage significantly promoted the shoot growth of pepper compared with low nitrogen treatments, while there were no significant differences in biomass and seedling quality index between high nitrogen and medium nitrogen (Table 4). These results indicated that higher nitrogen concentration after initiation of flower bud differentiation promoted the carbohydrate accumulation, plant biomass accumulation, and plant growth of pepper seedlings. In addition, plant roots are important organs for transporting water and nutrients of plants, and robust roots can transport enough water and nutrients for plant growth and development [38,39]. Proper nitrogen can lead to vigorous root growth in plants, and a large root absorption area with high root activity promoted plant growth, whereas low or high nitrogen concentration reduced the auxin concentration in roots and inhibited the root growth of plants [40,41].

4.2. Influences of Nitrogen Concentration at Different Growth Stages on Photosynthetic Pigment of Pepper Seedlings

Chlorophyll is closely related to photosynthesis of plants and participates in the energy conversion and substance conversion processes of plants, and chlorophyll content is related to plant nitrogen nutrition [42,43]. Previous studies have shown that low nitrogen supply reduced the chlorophyll synthesis in plant leaves and inhibited plant growth, while adequate nitrogen supply enhanced photosynthesis assimilation capacity and promoted plant growth [42,44]. In this study, under the condition of suitable nitrogen concentration in the second stage, the chlorophyll and carotenoid contents of pepper leaves under suitable nitrogen concentration in the first stage were significantly increased compared with those under low or high nitrogen concentration (Table 5). Our results indicated that the high photosynthetic pigment content in plant leaves under the appropriate nitrogen supply at the early growth stage may be due to the fact that the carbon and nitrogen metabolic balance of plants under the appropriate nitrogen treatment increased the photosynthetic pigment content. In addition, under the condition of suitable nitrogen concentration in the first stage, the chlorophyll and carotenoid contents of plant leaves under low nitrogen supply in the second stage were significantly reduced compared with those under higher nitrogen supply (Table 5). Our result suggested that the insufficient nitrogen supply after initiation of flower bud differentiation destroyed the chloroplast structure in the cell, inhibited the conversion of sugars into nitrogen compounds such as chlorophyll and amino acids, reduced the content of photosynthetic pigments, inhibited the photosynthesis of plants, and had negative impacts on plant growth [42,45].

4.3. Impacts of Nitrogen Concentration at Different Growth Stages on Enzyme Activity Related to Carbon and Nitrogen Metabolism of Pepper Seedlings

Carbon and nitrogen metabolism are the most important metabolic processes in the plant life cycle and are crucial for plant growth and development [46]. The activity of carbon and nitrogen metabolizing enzymes in plant leaves is significantly affected by nitrogen levels. Studies have shown that nitrogen deficiency affected the activities of enzymes related to nitrogen metabolism in plants, reduced the content of chlorophyll in leaves, and inhibited plant carbon metabolism [10,46,47]. Similarly, our results showed that the sucrose synthase, sucrose phosphate synthase, nitrate reductase, and glutamine synthetase activity in the leaves of pepper seedlings cultivated under appropriate nitrogen concentration in the first stage were significantly higher than those under low nitrogen concentration while the nitrogen concentration in the second stage was appropriate (Figure 2). In addition, the sucrose synthase, sucrose phosphate synthase, and nitrate reductase activity in the leaves of pepper seedlings grown under high nitrogen concentration in the second stage were significantly higher than those under low nitrogen concentration while the nitrogen concentration in the first stage was appropriate. Our results indicated that, in the early growth stage of pepper seedlings, nitrogen deficiency may significantly reduce the production of nitrate reductase mRNA and nitrate reductase activity in pepper leaves, leading to metabolic disorders of pepper seedlings, reduced carbon and nitrogen assimilation abilities, and decreased vegetative growth of plants [37]. However, appropriate nitrogen enhanced the carbon sequestration capacity and carbon metabolism of pepper leaves, which provided sufficient energy and carbon skeleton for the nitrogen metabolism of pepper leaves [46]. Additionally, appropriate nitrogen increased carbohydrate, amino acid, and protein concentrations, which provided necessary energy and material for plant growth and development [10,48].

4.4. Effects of Nitrogen Concentration at Different Growth Stages on Flower Bud Differentiation and Flowering of Pepper Seedlings

Flower bud differentiation is a key stage in the seedling growth process, which is related to seedling quality and plant yield [49]. In addition, the flower is the plant reproductive organ that determines plant fruit and yield, and early flowering is the key to early fruit [50,51]. Fertilization causes changes in metabolism and transport of carbohydrates in flower buds, which could affect flower bud differentiation and flowering [15,52]. Previous studies have shown that low nitrogen inhibited plant growth and resulted in less flowering, while the leaf growth and flowering of plants were promoted with a nitrogen concentration increase [17,18]. Similarly, under the moderate nitrogen concentration of the first stage, the flower buds and flowers of pepper seedlings were larger under the treatment of higher nitrogen concentration compared with those under low nitrogen concentration in the second stage (Figure 3). Under the moderate nitrogen concentration of the second stage, the flower buds of pepper seedlings were significantly larger, and the flower buds and flowers developed faster under the moderate nitrogen concentration compared with those under low or excess nitrogen concentration in the first stage. In addition, flower bud diameter was significantly positively correlated with shoot fresh weight, root fresh weight, shoot dry weight, seedling quality index, sucrose phosphate synthase activity, and glutamine synthetase activity (Figure 4). Our results showed that suitable nitrogen concentration at the early growth stage and higher nitrogen concentration after initiation of flower bud differentiation could promote leaf photosynthesis, promote the production, transport and accumulation of assimilation products, and provide sufficient nutrient accumulation for flower bud differentiation, thus promoting flower bud differentiation and flowering of plants [22]. However, excessive nitrogen concentration in early growth stage inhibited flower bud development, which may be due to the fact that excessive accumulation of reactive oxygen species caused by excessive nitrogen fertilizer influenced the normal physiological metabolism, led to C/N imbalance, and inhibited flower bud differentiation, restricting the expression of flowering related genes and the flowering of plants [28,48,53].

4.5. The Optimal Nitrogen Application Strategy of Different Growth Stages

The TOPSIS method is a multi-objective decision-making analysis method, and the traditional TOPSIS model has shortcomings in weight allocation. Therefore, an entropy weight method was used in this study to determine the objective weight of each evaluation index, which enhanced the scientific and rational evaluation results [12,54]. In this study, an entropy weight TOPSIS method was used to comprehensively evaluate the nitrogen concentration at different growth stages to determine the proper nitrogen management strategy for cultivating high-quality pepper seedlings. Our results showed that the T6 treatment ranked first, while the T1 treatments ranked ninth (Table 8). The results showed that the appropriate nitrogen concentration (15 mmol L^{-1}) at the early growth stage combined with higher nitrogen concentration (25.61 mmol L^{-1}) after initiation of flower bud differentiation had a positive impact and high feasibility for pepper seedling cultivation. Similar results were also found in tomato [9,12] and strawberry [17], where low or excessive nitrogen application reduced yield and water and nitrogen utilization rates; in addition, excessive nitrogen enriched nutrient content in the matrix and inhibited nutrient uptake by roots [12,17].

Regression models quantitatively describe statistical relationships, which are used to study causal relationships between variables. Dai et al. [55] and Wang et al. [56] used regression models to optimize water and nitrogen management of green pepper and cucumber under drip irrigation, respectively. Hao et al. [28] used regression analysis and response surface analysis to analyze the combination of light intensity and nitrogen concentration to regulate the yield and quality of purple cabbage. However, few studies establish a 3-D prediction model to investigate the influences of nitrogen concentration at different growth stages on seedling growth and flower bud differentiation of pepper seedlings. The regression model of our results indicated that the shoot fresh weight, root fresh weight, flower bud diameter, and glutamine synthetase activity of pepper seedlings grown under moderate nitrogen in the first stage combined with higher nitrogen in the second stage was high, which was suitable for the growth and development of pepper seedlings (Figure 5). The optical nitrogen fertilizer strategy suitable for pepper seedling cultivation was further verified by the establishment of a regression model, which provided guidance for pepper seedling cultivation.

5. Conclusions

Regulating nitrogen concentration at different plant growth stages coordinated the vegetative growth and reproductive growth of pepper seedlings. Our results indicated that moderate nitrogen concentration (15 mmol L^{-1}) in the first stage combined with high nitrogen concentration (25.61 mmol L^{-1}) in the second stage improved the carbon and nitrogen metabolism, increased the biomass accumulation, and promoted the vegetative growth and reproductive growth of pepper seedlings. Additionally, a regression model and a comprehensive evaluation model were established to determine the optimal nitrogen fertilizer supply strategy for pepper seedlings. Our study is of great significance for nitrogen fertilizer management at different growth stages and the cultivation of high-quality pepper seedlings. In addition, the endogenous hormones and relative expression levels of genes related to flower bud differentiation in pepper seedlings under nitrogen supply at different growth stages need to be further studied.

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