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Nitrogen Fertilization Improves Growth and Bioactive Compound Content for *Salvia miltiorrhiza* Bunge

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Abstract: *Salvia miltiorrhiza* B., an herb used in traditional Chinese medicine, has been widely used to prevent and treat cardiovascular and other diseases. Currently, the majority of medicinal plants used in the US are imported from foreign countries, which involves transportation, quality control, and other issues. The objective of this study was to investigate the effect of nitrogen fertilization on growth and content of tanshinone I, tanshinone IIA, cryptotanshinone, and salvianolic acid B for *Salvia miltiorrhiza* B. in Mississippi. Plants were fertilized with one of five nitrogen (N) rates (0, 2, 4, 6, or 8 g N/plant from NH_4NO_3). Plants were harvested in November 2020 and 2021. Plants treated with 8 g N had higher plant growth index, leaf SPAD value, shoot and root number, shoot and root weight, maximum root length and diameter, shoot: root ratio, N concentration in root, and content of bioactive components compared to plants treated with 0, 2, 4 g N. Plants receiving 6 g N had similar shoot number, maximum root length, maximum root diameter, root weight and content of bioactive components compared to plants receiving 8 g N. However, plants receiving 6 g N had higher photosynthetic activity compared to plants receiving the higher N rate. Higher N rates increased plant growth and content of tested bioactive compounds.

Keywords: danshen; nitrogen; tanshinone I; tanshinone IIA; cryptotanshinone; salvianolic acid

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1. Introduction

Danshen (*Salvia miltiorrhiza* B.) is in the family Lamiaceae. It is an important and popular traditional Chinese plant that has been cultivated for a long time in Asia for its medicinal value [1]. Danshen is an herbaceous perennial plant. In its natural environment, danshen grows on hillsides, meadows, and grassy places along stream banks and forest margins, between elevations of 90–1200 m [2]. Danshen prefers full sun to light shade, a rich, moist, and well-drained light sandy soil, and is naturally distributed in parts of Japan and China. It is cold hardy to approximately -10 °C, and can grow in USDA hardiness zones 5–9 [3]. The plant is multi-branched and can reach between 30–60 cm in height with a similar spread, producing blue-purple or white floral spikes extending about 30 cm above the foliage [2,4]. Danshen contains a variety of chemical components, which are mainly divided into two categories: one is the lipid-soluble component dominated by tanshinone diterpenes, and the other is the water-soluble component dominated by phenolic acid [5]. Clinical trials and pharmacological studies indicate tanshinone I, tanshinone IIA, cryptotanshinone, and salvianolic acid B are major bioactive components, and have been considered marker compounds [6,7]. These major compounds in danshen resulted in its antioxidant, anti-microbial, anti-virus, anti-cancer, and anti-inflammatory properties. Therefore, danshen has been widely used to prevent and treat cardiovascular diseases [2].

Currently, the majority of medicinal plants including danshen used in the United States are imported from foreign countries. However, long-distance transportation costs are high, transport is slow, and there are also quality concerns. With the projected strong market growth of medicinal plant-derived products, it is critical for US industries to have access to adequate supplies of high quality medicinal plants [8]. At present, there are few studies on the planting of danshen in the United States. This study lays a foundation for the future development of danshen in the United States.

Danshen has been used for many years in China, Japan, and other east Asian countries to prevent and treat ailments including neurological disease, cancer, inflammation, diabetes mellitus, liver diseases, renal diseases, and cardiovascular and cerebrovascular diseases [9–13]. With the increasing awareness and usage of herbal medicines worldwide, there has been an increasing interest in the use of medicinal plants for alternative and complementary medicines to prevent and treat diseases including cardiovascular diseases in the United States. The use of danshen as a natural product has grown substantially in the United States and Europe in recent years [14]. Cardiovascular disease is the most deadly and widespread non-communicable disease in the world, including cancer, especially for the elderly [15]. In 2020, there were 690,882 deaths due to heart disease in the United States, accounting for 20.6% of all deaths [16]. Danshen is usually applied clinically in the form of compound preparations, for example, Compound Danshen Dripping Pill or Dantonic® (T89) Capsule, which is a 2-herb composition medicine including danshen and *Panax notoginseng* (Burkill) F.H.Chen as main ingredients. T89 is the world's first compound Chinese herbal medicine that has passed the US Food and Drug Administration (FDA) regulated multi-center phase 3 clinical trial, and now is in the process of seeking drug approval from FDA [17]. T89 is currently sold in China, Vietnam, South Korea, India, and some other countries as a prescription drug to treat angina and coronary heart diseases.

Fertilization can improve soil fertility and increase crop yield per unit area. Commercial fertilizer is the most basic and important material input in agricultural production [18]. Without fertilizers, the world would not be able to meet its food production targets. Forty to 60% of world food production is accomplished using commercial fertilizers [19]. Nitrogen deficiency can lead to abnormal root–shoot ratios, short lateral branches, small leaves, chloroplast disintegration, and even plant death [20]. However, excessive nitrogen had a negative effect on root growth, lower dry matter of plants, and made plants weak [21]. The results of a container study showed that increasing N rate increased danshen biomass and root quality, the highest dry matter was observed when the NH_{4+}:NO_{3-} ratio was 75:25 [22]. Another study found that high concentrations of nitrogen fertilizer was beneficial to dry matter accumulation of danshen aerial parts, and low concentration of nitrogen fertilizer transferred the dry matter accumulation to underground roots [23]. Optimal fertilization management for danshen production in relationship to root yield and bioactive compound contents largely remains unclear.

The objective of this study was to investigate the effect of nitrogen fertilization on growth and bioactive compound content for *Salvia miltiorrhiza* B. in Mississippi.

2. Materials and Methods

2.1. Plant Materials and Cultivation

Danshen seeds were sourced from Shandong province in China (36°07′ N 120°38′ E) and stored at 4 °C before use. Seeds were sown in 128-cell trays in a greenhouse at Mississippi State University (MSU, 33°29′ N 88°47′ W) in March 2020 and 2021. One month after germination, the seedlings were transplanted into 0.5 L containers and continued to grow in the greenhouse. Plants were transplanted into 11.4 L containers in June 2020 and 2021, filled with a soilless substrate (Metro-Mix® 852, Sun Gro Horticulture, Agawam, MA, USA). Containers were then placed in full sun on a nursery pad at the MSU R. R. Foil Plant Science Research Center in Starkville, MS, USA. Danshen plants were subjective to five nitrogen fertigation treatments: 100 mL of nutrient solution containing 0, 2, 4, 6, or 8 g N per plant from NH_4 NO_3 once every two weeks. Other nutrients were supplied from N-free fertilizer (1.1 $mg \cdot mL^{-1}$, Cornell No N Formula 0-6-27, Greencare Fertilizers, Kankakee, IL, USA) (Available phosphate 6.8%, potash 27.1%, magnesium 4.7%, iron 0.134%, manganese 0.048%, zinc 0.005%, copper 0.002%, boron 0.048%, molybdenum 0.001%). Drip irrigation was installed, and plants were irrigated as needed. Plants were harvested on 2 December 2020 and 2021. The temperature in the greenhouse was set at 25 °C with natural light.

2.2. Plant Growth

Five plants from each nitrogen fertigation treatment were measured and harvested. Plant height, width 1, and width 2 were measured for all plants prior to harvest. Height was measured from the substrate surface to the top of the plant. Width 1 was measured at the greatest width of the plant and width 2 was the perpendicular width to width 1. Plant growth index (PGI) was calculated as the average of height, width 1, and width 2, i.e., PGI = [height + width 1 + width 2]/3. Relative leaf chlorophyll content measured as leaf SPAD were measured on three recent fully expanded leaves from each plant using the SPAD-502 meter (Konica Minolta, Inc., Osaka, Japan) on 2 December 2020 and 2021. The SPAD value of each plant was the average of the readings from three measurements [24]. Shoot number and shoot fresh weight were measured after harvest. Shoot number was counted as the number of primary shoots grown from the root. Root number, maximum root length, maximum root diameter, and root fresh weight were also measured. Root number was counted as the number of roots greater than 2 mm in diameter. Maximum root length was measured as the length of the longest root [25]. Maximum root diameter was measured as the diameter in the upper middle of the tap root [22]. Shoots and roots from each plant were then oven-dried at 60 °C until constant weight, and the dry weights of shoots and roots were measured.

2.3. Root Nitrogen Analyses

Dried root samples were ground to pass a 40-mesh (0.43 mm) sieve using a Wiley mill (Thomas Scientific, Thorofare, NJ, USA). Nitrogen concentration (%) was determined by Kjeldahl method [26].

2.4. Photosynthetic Activities

Plant photosynthetic activities of one randomly selected plant from each replication were measured on 10 September 2021, using a portable photosynthesis system (LI-6400 XT; LI-COR Biosciences, Lincoln, NE, USA) with the leaf chamber with a fluorometer light source. For each plant, one recent fully expanded leaf was enclosed into a 2 cm^2 leaf chamber for measurement. Photosynthetically active radiation (PAR) and reference CO_2 concentration inside the leaf chamber were maintained at 1500 $\mu mol \cdot m^{-2}\ s^{-1}$ and 400 $\mu mol \cdot mol^{-1}$ during sampling, respectively. The leaf chamber block temperature was maintained the same as the air temperature [27]. Leaf transpiration rate (Trmmol), net photosynthetic rate (Pn), intercellular CO_2 concentration (Ci), leaf-to-air vapor pressure deficit (VPDL), and stomatal conductance (g_s) were measured.

2.5. Preparation of Danshen Extract

Dried root samples were ground to pass a 40-mesh (0.43 mm) sieve using a Wiley mill (Thomas Scientific, Thorofare, NJ, USA). Dry root samples of 0.5 g were mixed with 50 mL 75% methanol solution in 50 mL conical flasks, and the flasks were then sealed. Sealed flasks containing the mixture were ultrasonically extracted for 30 min at room temperature. After ultrasonic extraction, the extracted solution was filtered through a filter paper (Grade 1, GE Healthcare Bio-Sciences Corp., Marlborough, MA, USA) using a vacuum pump, then fixed volume to 50 mL with 75% methanol solution in volumetric flasks [28,29]. The extractions were prepared for biochemical composition analysis including tanshinone I, tanshinone IIA, cryptotanshinone, and salvianolic acid B.

2.6. Analysis of Tanshinone I, Tanshinone IIA, Cryptotanshinone, and Salvianolic Acid B

Tanshinone I, tanshinone IIA, cryptotanshinone, and salvianolic acid B contents in danshen extract were analyzed using high performance liquid chromatography (HPLC) (1260 Infinity II series; Agilent Technologies, Wilmington, DE, USA). Danshen extract was filtered through a 0.22 μm membrane. HPLC analyses were performed using a diode array detector (G1315C Diode-array Detector, Agilent Technologies, Wilmington, DE, USA) with an injection volume of 10 μL, flow rate of 1 $mL\cdot^{-1}$, controlled oven temperature of 30 °C, and a C18 column [Agilent TC-C18 (2), 4.6 mm × 250 mm, 5 μm; Agilent Technologies, Wilmington, DE, USA]. Mobile phase A was 100% acetonitrile, mobile B was 0.02% phosphoric acid [30].

The elution program for tanshinone I, tanshinone IIA, and cryptotanshinone was 0–6 min: 61% mobile phase A and 39% mobile phase B; 6–20 min: percentage of mobile phase A linearly increased from 61 to 90%, percentage of mobile phase B linearly decreased from 39 to 10%; 20–20.5 min: percentage of mobile phase A linearly decreased from 90 to 61%, percentage of mobile phase B linearly increased from 10 to 39%; 20.5–25 min: 61% mobile phase A and 39% mobile phase B. Chromatograms were recorded at 270 nm. This procedure was modified slightly based on the method described in Chinese Pharmacopoeia [30].

The elution program for salvianolic acid B was 0–20 min: percentage of mobile phase A linearly increased from 5 to 20%, percentage of mobile phase B linearly decreased from 95 to 80%; 20–30 min: percentage of mobile phase A linearly increased from 20 to 30%, percentage of mobile phase B linearly decreased from 80 to 70%; 30–40 min: percentage of mobile phase A linearly increased from 30 to 40%, percentage of mobile phase B linearly decreased from 70 to 60%. Chromatograms were recorded at 280 nm. This method was modified slightly based on Ren's study [31].

Standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). The retention time of cryptotanshinone, tanshinone I and tanshinone IIA were 12.75, 14.07, and 17.33 min, and the retention time of salvianolic acid B was 36.14 min. Tanshinone I, tanshinone IIA, cryptotanshinone, and salvianolic acid B contents in danshen were calculated by standard curve. Mobile phases of HPLC grade were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.7. Experimental Design and Statistical Analysis

The experiment was a completely randomized design. Thirty plants were randomly assigned to one of five N rates. Significance of the main effect within year were determined by analysis of variance (ANOVA) using the PROC GLM procedure. Where indicated by ANOVA, means were separated by Tukey's honestly significant difference test at $p \leq 0.05$. Data from the two years were compared as repeated measures. All statistical analyses were performed using SAS (version 9.4, SAS Institute, Cary, NC, USA).

3. Results

3.1. Plant Growth Index, Leaf SPAD Values, Shoot Number, and Root Number

PGI, SPAD, shoot number, and root number generally increased with increasing N rate. Plants fertilized with 8 g N had greater PGI, SPAD values, and root numbers compared to plants receiving any other rate of fertilization, regardless of year (Table 1). Plants receiving 0 g N consistently had the lowest PGI or SPAD values compared to all other fertilization rates, regardless of year. Shoot numbers were greatest, ranging from 10.3 to 11.8 per plant, for plants receiving 6 or 8 g N compared to plants receiving less N although there were no differences between the two highest rates, regardless of year.

Table 1. Plant growth index (PGI), leaf SPAD value, shoot number, and root number of plants receiving five N rates in 2020 and 2021.

N Rate (g)	**PGI** [1]		**SPAD**		**Shoot Number (Per Plant)**		**Root Number (Per Plant)** [2]	
	2020	**2021**	**2020**	**2021**	**2020**	**2021**	**2020**	**2021**
0	22.8 ± 2.8 d [3]	23.4 ± 1.7 d	16.3 ± 2.6 d	12.3 ± 1.1 e	4.8 ± 0.8 c	5.0 ± 0.7 c	20.0 ± 1.2 c	21.8 ± 3.0 d
2	31.4 ± 3.8 c	35.1 ± 3.0 c	22.0 ± 2.2 c	22.1 ± 2.3 d	8.0 ± 1.0 b	5.8 ± 0.8 bc	33.8 ± 4.6 b	37.8 ± 3.6 c
4	36.5 ± 4.7 c	39.1 ± 3.7 c	23.6 ± 3.0 c	25.4 ± 2.2 c	8.4 ± 1.3 b	7.0 ± 0.7 b	37.8 ± 2.5 b	39.8 ± 3.1 bc
6	50.7 ± 4.6 b	54.9 ± 4.1 b	29.9 ± 3.2 b	31.0 ± 2.9 b	10.8 ± 1.3 a	10.3 ± 1.1 a	38.6 ± 3.8 b	44.6 ± 4.0 b
8	56.0 ± 4.0 a	61.7 ± 6.2 a	35.7 ± 7.3 a	35.9 ± 3.3 a	11.8 ± 0.8 a	11.6 ± 1.1 a	62.6 ± 5.9 a	55.2 ± 4.9 a
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1] Plant growth index (PGI) was calculated as the average of plant height, width 1 (widest points apart), and width 2 (perpendicular to width 1). [ph + width 1 + width 2]/3. [2] Root number was counted as the number of roots greater than 2 mm in diameter. [3] Different lower-case letters within a column suggest significant differences among N rates indicated by Tukey's HSD test at $p \leq 0.05$. Data are given as mean ± S.D.

3.2. Shoot Fresh Weight, Dry Weight, Maximum Root Length, and Maximum Root Diameter

Plants receiving 8 g N had higher shoot FW and DW compared to all other treatments, regardless of year (Table 2). Plants receiving 6 or 8 g N had greater maximum root lengths in 2020 and maximum diameters, regardless of year, compared to all other N treatments. However, maximum root lengths were similar for plants receiving 4, 6, or 8 g N in 2021.

Table 2. Shoot fresh weight, dry weight, maximum root length, and maximum root diameter of plants receiving five N rates in 2020 and 2021.

N Rate (g)	**Shoot Fresh Weight (g Per Plant)**		**Shoot Dry Weight (g Per Plant)**		**Maximum Root Length (cm)** [1]		**Maximum Root Diameter (mm)** [2]	
	2020	**2021**	**2020**	**2021**	**2020**	**2021**	**2020**	**2021**
0	27.0 ± 1.1 e [3]	26.3 ± 3.2 e	10.4 ± 0.8 e	10.8 ± 1.3 e	35.2 ± 1.8 c	33.6 ± 2.3 b	7.4 ± 1.5 c	8.8 ± 0.4 c
2	57.7 ± 1.2 d	53.4 ± 3.0 d	16.7 ± 1.3 d	16.3 ± 0.8 d	42.8 ± 3.1 b	34.0 ± 2.6 b	10.6 ± 1.6 b	9.7 ± 1.0 c
4	64.0 ± 2.2 c	65 ± 2.8 c	19.9 ± 1.8 c	20.6 ± 1.9 c	45.2 ± 1.5 b	45.2 ± 2.2 a	11.7 ± 1.5 b	12.5 ± 1.2 b
6	103.7 ± 3.9 b	103.3 ± 5.9 b	25.2 ± 2.8 b	24.3 ± 1.9 b	48.4 ± 1.1 a	45.4 ± 2.3 a	14.4 ± 1.7 a	15.2 ± 0.7 a
8	125.6 ± 2.7 a	124 ± 3.5 a	27.6 ± 1.8 a	27.8 ± 1.4 a	50.0 ± 1.7 a	47.0 ± 4.3 a	14.4 ± 1.5 a	15.9 ± 0.6 a
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1] Maximum root length was measured as the length of the longest root. [2] Maximum root diameter was measured as the diameter of the thickest root. [3] Different lower-case letters within a column suggest significant differences among N rates indicated by Tukey's HSD test at $p \leq 0.05$. Data are given as mean ± S.D.

3.3. Root Fresh Weight, Root Dry Weight, Shoot: Root Ratio, and N Concentration in Root

Danshen fertilized with 6 or 8 g N had greater root fresh and dry weights than plants receiving any other level of N fertilization, regardless of year (Table 3). Plants receiving 2 or 4 g N had greater root fresh or dry weight compared to plants receiving 0 g N. Shoot: root ratios were greatest for plants receiving 8 g N compared to all other treatment levels, and plants receiving 0 g N had the lowest shoot: root ratio, regardless of year. N concentration in root were also greatest in plants receiving 8 g N compared to all other fertilization levels, regardless of year. Plants receiving 4 or 6 g N had similar root N levels in 2021, but plants receiving 6 g N had greater N concentration in root compared to plants receiving 4 g N in 2020.

Table 3. Root fresh, dry weight, shoot: root ratio, and N concentration in root of plants receiving five N rates in 2020 and 2021.

N Rate (g)	Root Fresh Weight (g Per Plant)		Root Dry Weight (g Per Plant)		Shoot:Root Ratio		N Concentration in Root (%)	
	2020	2021	2020	2021	2020	2021	2020	2021
0	110.2 ± 6.7 c [1]	109.4 ± 7.2 c	43.6 ± 1.4 c	42.8 ± 2.0 c	0.24 ± 0.02 d	0.24 ± 0.02 e	0.75 ± 0.12 e	0.81 ± 0.07 d
2	205.6 ± 21.4 b	201 ± 12.7 b	64 ± 2.7 b	65.8 ± 1.0 b	0.29 ± 0.01 c	0.27 ± 0.01 d	0.9 ± 0.05 d	1.02 ± 0.08 c
4	210.1 ± 25.4 b	204.2 ± 16.4 b	68.4 ± 1.9 b	68 ± 2.0 b	0.31 ± 0.03 c	0.32 ± 0.01 c	1.1 ± 0.13 c	1.18 ± 0.1 b
6	289.2 ± 25.1 a	281.8 ± 14.1 a	87.6 ± 1.7 a	88.1 ± 1.6 a	0.34 ± 0.02 b	0.37 ± 0.01 b	1.23 ± 0.08 b	1.27 ± 0.1 b
8	311.5 ± 28.2 a	294 ± 19.7 a	92.1 ± 7.6 a	88.8 ± 5.6 a	0.37 ± 0.02 a	0.42 ± 0.02 a	1.4 ± 0.1 a	1.5 ± 0.11 a
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1] Different lower-case letters within a column suggest significant differences among N rates indicated by Tukey's HSD test at $p \leq 0.05$. Data are given as mean ± S.D.

3.4. Photosynthetic Activities

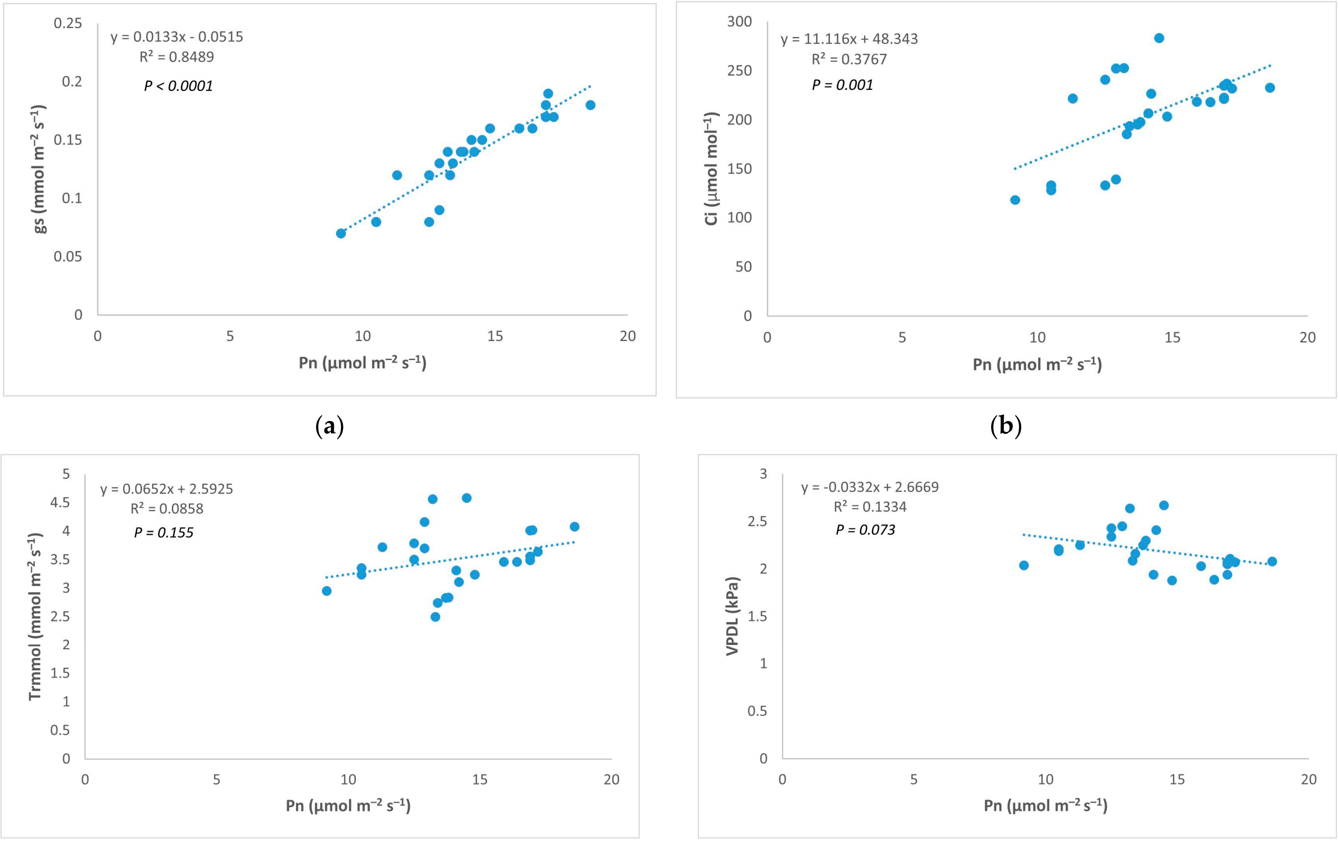
Net photosynthesis (Pn) was greatest for plants receiving 4 or 6 g N of 16.8 μmol m^{-2} s^{-1} and 16.2 μmol m^{-2} s^{-1} compared to plants receiving any other level of fertilization (Table 4). Plant receiving 2 or 8 g N had similar Pn although both had greater Pn levels compared to plants receiving 0 g N. Stomatal conductance (g_s) was greatest for plants receiving 4 or 6 g N and lowest for plants receiving 0 g N of compared to all other fertilization rates. Plants receiving 4 g N had higher g_s compared to plants receiving 2 or 8 g N, which were similar. Intracellular CO_2 concentration (Ci) and leaf transpiration rates (Trmmol) had a similar pattern. Plants fertilized with 2 g N had greater Ci and Trmmol levels compared to any other treatment. Plants receiving 4 or 6 g N had similar Ci and Trmmol levels but higher compared to plants receiving 8 g N. Plants receiving 0 g N had the lowest Ci and Trmmol levels. Vapor pressure deficits were greatest for plants receiving 2 g N compared to plants receiving any other N rate. Plants receiving 0 or 8 g N had similar VPDL levels, but higher than plants receiving 4 or 6 g N.

Table 4. Photosynthetic activities of plants receiving five N rates in 2021.

N Rate (g)	Pn [2]	g_s [3]	Ci [4]	Trmmol [5]	VPDL [6]
	(μmol m^{-2} s^{-1})	(mol m^{-2} s^{-1})	(umol mol−1)	(mmol m^{-2} s^{-1})	(kPa)
0	11.1 ± 1.6 c [1]	0.08 ± 0.007 c	130.7 ± 7.9 d	3.35 ± 0.28 b	2.25 ± 0.16 b
2	12.9 ± 1.2 b	0.132 ± 0.009 b	250.32 ± 22.29 a	4.16 ± 0.41 a	2.49 ± 0.17 a
4	16.8 ± 1.4 a	0.17 ± 0.02 a	224.04 ± 12.43 b	3.67 ± 0.33 b	2.04 ± 0.06 c
6	16.2 ± 1.2 a	0.168 ± 0.008 a	221.71 ± 12.02 b	3.58 ± 0.31 b	1.97 ± 0.1 c
8	13.7 ± 0.4 b	0.134 ± 0.01 b	199.9 ± 15.8 c	2.8 ± 0.22 c	2.24 ± 0.12 b
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1] Different lower-case letters within a column suggest significant differences among N rates indicated by Tukey's HSD test at $p \leq 0.05$. Data are given as mean ± S.D. [2] Net photosynthesis (Pn). [3] Stomatal conductance (g_s). [4] Intracellular CO_2 concentration (Ci). [5] Leaf transpiration rates (Trmmol). [6] Leaf-to-air vapor pressure deficits (VPDL).

The g_s was strongly positively linearly correlated with Pn. Ci and Trmmol had relatively weak positive linear correlations with Pn. In contrast, VPDL had negatively linear correlation with Pn (Figure 1).



(**a**) (**b**)

(**c**) (**d**)

Figure 1. Correlation of photosynthesis. (**a**) Correlation between net photosynthesis (Pn) and stomatal conductance (g_s); (**b**) Correlation between net photosynthesis (Pn) and intracellular CO_2 concentration (Ci); (**c**) Correlation between net photosynthesis (Pn) and leaf transpiration rates (Trmmol); (**d**) Correlation between net photosynthesis (Pn) and leaf-to-air vapor pressure deficit (VPDL).

3.5. Tanshinone I, Tanshinone IIA, Cryptotanshinone, and Salvianolic Acid B

Levels of all bioactive compounds were greatest for plants receiving 6 or 8 g N compared to plants receiving lower rates of N, regardless of year (Table 5). Beginning with plants receiving 4 g N, tanshinone I, tanshinone IIA, and salvianolic acid B levels declined significantly with each progressive decrease in N levels, regardless of year, and were lowest for plants receiving 0 g N compared to any other fertilization level. Cryptotanshinone levels followed a similar trend in 2021, but plants receiving 2 or 4 g N had similar cryptotanshinone levels in 2020.

Table 5. Content of tanshinone I, tanshinone IIA, cryptotanshinone, and salvianolic acid B in roots of plants receiving five N rates in 2020 and 2021.

N Rate (g)	Tanshinone I (%)		Tanshinone IIA (%)		Cryptotanshinone (%)		Salvianolic Acid B (%)	
	2020	2021	2020	2021	2020	2021	2020	2021
0	0.02 ± 0.005 d [1]	0.014 ± 0.002 d	0.215 ± 0.007 d	0.225 ± 0.005 d	0.079 ± 0.011 c	0.073 ± 0.007 d	2.425 ± 0.063 d	2.399 ± 0.036 d
2	0.05 ± 0.006 c	0.053 ± 0.003 c	0.243 ± 0.01 c	0.247 ± 0.007 c	0.106 ± 0.017 b	0.1 ± 0.006 c	2.849 ± 0.111 c	2.799 ± 0.11 c
4	0.061 ± 0.001 b	0.065 ± 0.004 b	0.315 ± 0.01 b	0.308 ± 0.011 b	0.118 ± 0.008 b	0.119 ± 0.008 b	3.304 ± 0.223 b	3.269 ± 0.231 b
6	0.077 ± 0.004 a	0.082 ± 0.007 a	0.344 ± 0.008 a	0.346 ± 0.007 a	0.153 ± 0.012 a	0.159 ± 0.005 a	3.926 ± 0.193 a	3.855 ± 0.099 a
8	0.078 ± 0.005 a	0.078 ± 0.006 a	0.339 ± 0.01 a	0.344 ± 0.013 a	0.143 ± 0.01 a	0.158 ± 0.011 a	3.868 ± 0.101 a	3.848 ± 0.06 a
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1] Different lower-case letters within a column suggest significant differences among N rates indicated by Tukey's HSD test at $p \leq 0.05$. Data are given as mean ± S.D.

4. Discussion

In general, N plays an important role in the growth of plants [32]. Danshen is generally regarded as a plant that can be greatly affected by N [22,33]. Higher N rate generally increases the growth of aboveground parts of danshen [23], which agrees with our results. In this study, 8 g N produced plants with greater PGI, SPAD, shoot number, shoot fresh weight, shoot dry weight, shoot: root ratio, and photosynthetic rate. Our results demonstrating the relationship between N rate and growth of aboveground plant parts were also reported by Sheng [25]. Other medicinal plants such as *Cyamopsis tetragonoloba* L. and *Echinacea purpurea* L. have demonstrated a similar relationship [34,35]. In another study, 100 and 150 ppm N were considered beneficial for nitrogen fixation, absorption of nutrients, and secretion of growth substances such as auxins, gibberellins, cytokinins of the strawberry under hydroponic system [36]. In addition, the phosphorus (P) content in plant tissues from higher N treatments was significantly higher compared to plants receiving lower N treatments in this study (data not shown), indicating that N was helpful for the absorption of phosphorus, which was consistent with the results of Sheng and Chowdhury and Rosario [25,37]. Nitrogen is thought to contribute to the synthesis of organic compounds such as proteins, amino acids, enzymes, and nucleic acids. Phosphorus is considered to be a component of enzymes, phospholipids and other substances involved in the activation and transport of organic materials for growth and metabolism [38]. N and P also promote shoot growth synergistically [39]. In this study, 8 g N rate increased SPAD values, agreed with Reyes et al. reporting that SPAD value was positively correlated with chlorophyll content and N level [40], which could affect biomass and photosynthetic rate. In addition to directly increasing plant biomass, N has been reported to aid the basal lateral branches and leaf growth [25,41], which is consistent with our results. According to our results, danshen fertilized with 8 g N had the greatest PGI, SPAD value, shoot fresh weight, shoot dry weight, and root N concentration, regardless of year. Shoot numbers were similar in plants fertilized with 6 and 8 g N, regardless of year.

Photosynthesis is the process by which plants convert solar energy into biological energy and a complex process affected by many factors [42]. Photosynthesis has been reported to be strongly influenced by nitrogen [43]. Shangguan et al. [44] reported that winter wheat fertilized with level of N (15 mmol/L N) had significantly improved net photosynthetic rates compared with plants receiving lower N levels. In this study, N levels affected both Pn and g_s of danshen. The net photosynthetic rate of danshen fertilized with 4 or 6 g N was the highest, indicating that danshen needs sufficient levels of N fertilizer to maintain photosynthesis. However, excessive nitrogen may inhibit photosynthesis. Cun et al. [45] found the higher N fertilizer inhibited the photosynthetic performance of *Panax notoginseng* (Burkill) F.H.Chen. There are many factors that affect photosynthetic characteristics, such as species, temperature, and moisture [42]. Cechin et al. [46] reported N had no effect on the g_s of *Helianthus* L., but Zhu et al. [47] reported N significantly increased g_s in Manchurian ash and Mongolian oak. Plants fertilized with 4 or 6 g N has the highest g_s in this study. Ci, Trmmol and VPDL are also considered nitrogen-influenced measurements [46,48]. According to the results of this study, the highest Ci, Trmmol and VPDL of danshen resulted from 2 g N, indicating that the Ci, Trmmol and VPDL of danshen could be reduced by higher nitrogen levels.

In this study, the highest two N levels increased root biomass. This result was similar to that of Sheng [25] and Han [49], where root growth was significantly increased under high N and high P mixed treatment. Regardless of year, the largest root fresh and dry weights were found in plants fertilized with 6 g or 8 g N. The increase in root weight was obviously the result of increase in root number, root length, and root diameter. Walch-Liu et al. [50] reported lateral root growth was enhanced by nutrients in the soil, especially N, which is consistent with our results. Cotton was reported to have significantly increased root length, root surface area, and root biomass in most soil layers at appropriate N rate (240 kg·HA^{-1}) compared with the N rate (0 kg·HA^{-1}) [51]. In this study, the 8 g and 6 g N treatments resulted in the largest root number, maximum root length, and maximum root diameter. It was indicated that danshen is one of the plants that require at least 6 g N per plant per year [23]. Combined with previous studies and the results of this study, the increase in photosynthetic rate sustained by appropriate nitrogen application rate may have promoted root growth [52]. The shoot: root ratio of plants changed with the N application rate, reflecting the N utilization efficiency of plants [53]. According to our results, the shoot: root ratio increased with the increase of nitrogen application, indicating that nitrogen promoted the stem growth more than the root, and the same results have been reported in previous studies [22,25]. The root biomass of danshen is generally of greater concern, and excessive N fertilizer may not potentially increase the root biomass of danshen.

During the two years results of this study, higher N levels significantly increased the contents of tanshinone I, tanshinone IIA, cryptotanshinone and salvianolic acid B. This result is similar to some results of Wang's research, which indicated NH_4NO_3 fertilization promoted the best hairy root yield and content of bioactive components of *Salvia miltiorrhiza* B. and *Salvia castanea* D. [54]. The combination of N and P has also been reported to promote the accumulation of bioactive components in danshen [55,56], as mentioned above, N also promoted P uptake in roots in this study. Photosynthesis is a crucial factor that affects the production of bioactive components in plants. The photosynthetic rate determines the amount of energy that is available to the plant for growth and the production of bioactive components. When the photosynthetic rate is high, the plant has enough energy and photosynthetic product, which can supply the production of bioactive compounds [42]. In this study, Pn of plants receiving 6 g N was accompanied by higher contents of tanshinone I, tanshinone IIA, cryptotanshinone, and salvianolic acid B, which is similar to Zeng's results [57]. However, in the range of 0 g to 6 g N rate, a negative correlation between N levels and the contents of bioactive components of danshen was previously reported [22]. Because the bioactive components of danshen mainly relies on photosynthesis to produce organic matter to accumulate more bioactive components, this result may be due to differences in the space in which the aboveground part of the plant grows [22]. In addition, in this study, the N concentration in root between 6 and 8 g N has significant differences in both years, but the content of bioactive components between 6 and 8 g N have no significant differences in two years. This result shows that 8 g N can continue to increase N concentration in root, but it cannot continue to help plants produce higher contents of bioactive components. The content of bioactive components is an important indicator of the quality of danshen. Excessive N did not increase the content of bioactive components, which is similar to Sheng's results [25]. Plant cultivar, climate, temperature, and geographical characteristics have all been reported to influence the quality of medicinal plants [58–60], all these reasons may be potential influencing factors.

5. Conclusions

In conclusion, nitrogen rate of 8 g per plant resulted in danshen plants with the greatest PGI, shoot number, root number, shoot weight, maximum root length, maximum root diameter, root weight, SPAD value, shoot: root ratio, N concentration in root and contents of bioactive components. Plants receiving 6 g N produced similar shoot number, maximum root length, maximum root diameter, root weight and contents of bioactive components, and the highest rates of Pn and g_s. Based on the N rates tested in this study, 6 g N is recommended for container cultivation of danshen in Mississippi. Further research on optimal rates of N and other nutrients will help develop sustainable fertilizer management practices to optimize danshen quality while reducing fertilizer input.

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