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Original Research

Growth and Physiological Changes of *Juglans Regia* L. Seedlings under Nitrogen Deficiency Stress

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Abstract

Walnut (*Juglans regia* L.) seedlings were treated with varying degrees of nitrogen (N) deficiency. The effects of N deficiency on the growth and physiology of walnut seedlings were investigated, and the adaptability and mechanism of walnut seedlings to the N-deficient environment in barren soil were explored. The purpose is to provide a scientific basis for the breeding and fertilization management of walnut varieties with high resistance and to promote the healthy and sustainable development of the walnut industry. The results showed that: (1) Under N-deficient conditions, the aboveground biomass, root biomass, chlorophyll *a*, chlorophyll *b*, and carotenoid contents of walnut seedlings were clearly lower than those of the control group, and generally diminished more obviously with the aggravation of N deficiency and the prolongation of treatment time; (2) The net photosynthetic rate, stomatal conductance, and transpiration rate of walnut seedlings in the control (CK) group were the highest overall across all N deficiency, followed by the moderate N deficiency (MN) group and the lowest in the severe N deficiency (SN) group; (3) Under N deficiency, the contents of indole-3-acetic acid (IAA), cytokinin (CTK), and abscisic acid (ABA) in the walnut seedlings increased significantly, and the contents in SN group were the highest. With processing time extended, the contents of IAA and ABA in walnut seedlings under N deficiency increased continuously, while CTK content increased rapidly within 30 d, and then declined speedily after 30 d. In addition, the ethylene content of walnut seedlings under N deficiency stress gradually increased within 30 d, and then dropped dramatically after 30 d, which was generally lower than that of the CK group; (4) During the whole experiment, the contents of putrescine (Put), spermidine (Spd), and spermine (Spm) in walnut seedlings under N deficiency stress were lower than those in the CK group in general. This indicated that the accumulation of Put, Spd, and Spm in leaves was inhibited under N deficiency; (5) Compared with the CK group, the root activity

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of the SN group lessened obviously, while that of MN group was slightly higher than CK group on the whole. Based on the above results, this paper concluded that under N deficiency stress conditions, walnut seedlings could strengthen root vigor by regulating hormone content, accelerating cell division and increasing root nutrient supply, thereby alleviating the stress. However, long-term N deficiency could result in the disorder of hormones and polyamine synthesis in walnut seedlings, impairing photosynthesis and slowing down root growth, thus affecting the growth of the entire seedlings and leading to seedling aging.

Keywords: climate crisis, sustainable agricultural development, multistage dynamic DID model, policy implications, mechanisms

Introduction

Walnut (*Juglans regia* L.) ranks as one of the four major dried fruits in the world along with almond, cashew and hazelnut in the international market. It is also one of the four major woody oil plants in China, which has high nutritional and healthcare value, and is favored by domestic and foreign consumers. In recent years, China has issued a series of guiding documents and policies to increase the supply of healthy and high-quality edible vegetable oil and safeguard national food safety. The rapid development of the walnut-based woody oil plant industry is the direct result of this effort. At present, the cultivation area of walnuts in China has reached up to 50 million mu, ranking first in the world [1]. However, with the expansion of walnut planting area, the management level of growers has been gradually lowered, especially the weak awareness of scientific fertilization, which leads to the deterioration of soil structure, and problems of nutrient depletion and imbalance abound. It is not uncommon for plants to suffer from stunted growth and reduced plant yield and quality due to the lack of specific nutrient elements. In this case, the ideal variety characteristics could not be obtained to reap the expected economic benefits, which seriously restricts the development of the walnut industry in China [1-2].

As the major mineral nutrient necessary for plant growth, nitrogen (N) is an essential constituent of proteins, nucleic acids, chlorophyll and some hormones in plants, and it plays a crucial role in photosynthesis, respiration, protein synthesis and fat metabolism [3-4]. Long-term N deficiency can severely affect the growth and development of plants, such as allergic reactions in leaves, variation in nutrient absorption and accumulation in different parts of plants, stunted root morphological development, reduced biomass accumulation, and diminished flowering and seed setting rates [5]. These findings have been observed in *Eucalyptus* [6], bergamot pear [7], sandalwood [8], *Calophyllum inophyllum* [9], wheat [10], and cucumbers [11]. Plants absorb N from the soil, but in natural conditions, the source of soil N is limited. At present, soil N deficiency has become one of the primary factors restricting plant growth and development worldwide [12]. Many scholars believe that to solve the problem of insufficient soil N supply, it is necessary to understand the absorption and utilization of N by plants under low soil N conditions, to tap the potential of plant growth and low N tolerance, and ultimately to improve the growth performance of plants under low N stress [13]. In China, an enormous amount of research effort goes into genetics and breeding, nutrient and chemical compositions, and the development and utilization of walnuts. However, there are few studies on nutrient deficiency stress and response mechanism of walnuts [14]. In this study, the effect of the N deficiency stress on the growth and physiology of the walnut seedlings was investigated, and the adaptability of the walnuts under the N deficiency stress and its mechanism were explored, in order to provide reference for the breeding of walnut varieties with high resistance and fertilization management, thereby promoting the healthy and sustainable development of the walnut industry.

Materials and Methods

Experimental Materials and Design

Healthy walnut seedlings, Yucheng No. 1, were selected for the experiment and purchased from Chongqing Yulu Forestry Development Co., Ltd. Walnut seeds were stored in the sand in the greenhouse of Chongqing Academy of Forestry in late November, 2018. At the beginning of February, 2019, seeds were sown in seedbeds containing substrate (peat: yellow soil: perlite = 5: 4: 1) and routine management was conducted. In early April, 2019, healthy young seedlings at similar stages of growth (height 20 cm, ground diameter about 3 mm) were selected, rinsed with deionized water, and planted in ceramic pots (height 20 cm, diameter 18 cm) with clean quartz sand. Each pot was filled with 5 kg of sand, and a total of 90 seedlings were planted. There was a 15-day seedling recovery period after transplanting, during which only conventional water management was performed. Subsequently, the seedlings were randomly divided into three groups of 30 pots each. Different nutrient treatments were carried out on the basis of the Hoagland solution: control group (CK), complete nutrient solution; medium N treatment (MN), in which the N content was 50% of the complete nutrient solution;

Table 1. Experiment scheme of nutrient deficiency of the walnut seedlings.

Nutrient conditions CK		Treatment		
		MN	SN	
Macro element (mg L^{-1})	$Ca(NO_3)_2 \cdot 4H_2O$	945	472.5	—
	KNO_3	607	303.5	—
	$NH_4H_2PO_4$	115	57.5	—
	$MgSO_4$	493	493	493
	$CaCl_2$	—	222.2	444.5
	KCl	—	223.7	447.3
	$NaH_2PO_4 \cdot H_2O$	—	70.85	141.7
Iron salt (pH = 5.5)/(g L^{-1})	$FeSO_4 \cdot 7H_2O$	5.56	5.56	5.56
	Na_2EDTA	7.46	7.46	7.46
Micro element (pH = 6.0)/(mg L^{-1})	KI	0.83	0.83	0.83
	$MnSO_4$	22.3	22.3	22.3
	Na_2MoO_4	0.25	0.25	0.25
	$CuSO_4$	0.025	0.025	0.025
	$CoCl_2$	0.025	0.025	0.025
	H_3BO_3	6.2	6.2	6.2
	$ZnSO_4$	8.6	8.6	8.6

severe N deficiency treatment (SN), in which the N content was 0% of the complete nutrient solution. The scheme of each nutrient treatment is shown in Table 1. The amount of nutrient solution applied in each treatment during cultivation was determined according to the pre-experiment. The seedling roots were uniformly irrigated with a specific amount of nutrient solution at intervals of 5 d using a sprinkling, 100 mL per pot, during which routine water management was performed. The growth and physiological indicators of seedlings were measured every 15 d.

Experimental Method

Biomass Determination

Five seedlings were selected randomly for each treatment (i.e. five replicates, the same below). The entire seedlings were harvested, and the aboveground parts were separated from the roots. After washing with deionized water, the two parts were dried in an oven at 80ºC to constant weight. Their dry weight was weighed out on a one hundred thousandth electronic balance. Root: shoot ratio = root biomass/aerial biomass.

Determination of Photosynthetic Pigment Content

The photosynthetic pigments in leaves were extracted with pigment extraction solution (acetone: ethanol: water = 4: 5: 1) to determine their content. The last 2-3 climax leaves (the same below) were selected, 0.1 g of each leaf sample was weighed, and 10 mL of the acetone/ethanol mixture was added for extraction for 24 h. The extracted solutions were colorized at wavelengths of 470 nm, 645 nm, and 663 nm, respectively. The contents of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids (Car) were estimated by colorimetry. Chlorophyll *a/b* ratio (Chl *a/b*) = Chl *a*/Chl *b*.

Determination of Photosynthetic Parameters

Photosynthetic parameters were measured from 9:00 to 11:00 on sunny days. Net photosynthetic rate (*Pn*), stomatal conductance (g*s*) and transpiration rate (*Tr*) were determined using the LI-6800 portable photosynthesis system (LI-COR, USA). During the measurement, the indoor light intensity was set to 1200 μmol m^{-2} s^{-1}. The CO_2 concentration is the same as the atmospheric concentration, and the leaf temperature is 25~30ºC. Water use efficiency is defined as the amount of carbon assimilated as biomass per unit weight of water consumed. It is expressed as net photosynthetic rate/the transpiration rate.

Determination of Endogenous Hormone Content

Indole-3-acetic acid (IAA), cytokinin (CTK) and abscisic acid (ABA) were extracted according to the method of Feng et al. [15] and analyzed by an Agilent

1100 series HPLC system (Agilent Technologies Inc., USA) under the following conditions: the detection wavelength of IAA and ABA was 210 nm, and the detection wavelength of CTK was 265 nm; the injection volume was 20 μL; mobile phase methanol: acetonitrile: phosphate buffer (pH 3.5) = 15: 20: 65; column temperature was 35ºC; flow rate was 10 mL/min. Ethylene was extracted from leaves according to the method of Liu et al. [16]. It was detected by Shimadzu GC-9A gas chromatograph (Shimadzu Inc., Japan) under the following conditions: chromatographic column hp-5 (30×0.25 mm) and a detector FID (flame ionization detector) were used, the injection volume was 5 μL, and the detector temperature was 230ºC.

Determination of Polyamine Content

Putrescine (Put), spermine (Spm), and spermidine (Spd) were extracted according to the method of Hu et al. [17] and detected by the Agilent 1100 HPLC system (Agilent Technologies Inc., USA), under the following conditions: detection wavelength 254 nm, injection volume 10 μL, mophie phase 64% methanol (prepared in ultrapure water), column temperature 25ºC, flow rate 0.8 mL min^{-1}.

Determination of Root Activity

Root activity was determined by the TTC method [18]. Colorimetry was performed using a Shimadzu UV-2600 spectrometer (Shimadzu Inc., Japan) at the wavelength of 485 nm. The results of the blank experiment (firstly, sulfuric acid was added to inhibit TTC reduction caused by the dehydrogenase in the root system, and the rest of the procedures were the same as those in other treatments) were used as reference for measuring absorbance. Root activity was estimated by a standard curve.

Statistical Analysis

Microsoft Excel was used for data statistics and graphing. SPSS 20.0 was used for analysis of variance and correlation analysis.

Results and Discussion

Biomass Allocation of Walnut Seedlings under Different Treatments

As shown in Table 2, under N deficiency treatment, the biomasses of the aboveground part and root of walnut seedlings lessened clearly compared with the CK group, and declined more obviously with the increase of N deficiency level and the prolongation of fertilization time. All seedlings continued to grow under different degrees of N deficiency, but the growth rate was markedly lower than CK group, especially in the late stages of treatment (45-75 d). On the 75th d, the aboveground biomass of the MN group and the SN group was 41.2% and 69.2% lower than that of the CK group, respectively, and the difference was statistically significant ($P<0.05$). The root biomass of MN group and SN group was 18.3% and 37.2% lower than that of CK group, respectively, with statistical significance ($P<0.05$). On the contrary, with the increase of N deficiency level and the extension of treatment time, the root: shoot ratio of walnut seedlings generally increased gradually. On the 75th day, the root: shoot ratio of MN group and SN group was 39.2% and 105.1% higher than that of CK group, respectively, with significant deviation ($P<0.05$).

Table 2. Biomass allocation of walnut seedlings with different treatments (mean±SE).

Indicators	Treatment	Treatment time					
		0 d	15 d	30 d	45 d	60 d	75 d
Aboveground biomass (g)	CK	1.58±0.09 Fa	3.13±0.18 Ea	5.95±0.24 Da	11.38±0.59 Ca	17.43±0.83 Ba	24.61±1.69 Aa
	MN	1.63±0.08 Fa	2.09±0.12 Eb	4.14±0.27 Db	9.34±0.43 Cb	13.24±0.64 Bb	14.48±1.12 Ab
	SN	1.59±0.09 Ea	2.28±0.11 Db	3.54±0.19 Cc	6.34±0.32 Bc	7.28±0.46 Ac	7.57±0.63 Ac
Root biomass (g)	CK	1.12±0.08 Fa	3.17±0.21 Ea	3.76±0.20 Da	8.84±0.45 Ca	16.34±0.85 Ba	23.97±1.83 Aa
	MN	1.21±0.10 Fa	1.63±0.15 Eb	3.21±0.16 Db	8.14±0.39 Ca	13.25±0.69 Bb	19.59±1.18 Ab
	SN	1.17±0.09 Fa	1.59±0.13 Eb	3.38±0.18 Db	5.13±0.23 Cb	8.78±0.52 Bc	15.05±1.13 Ac
Root: shoot ratio	CK	0.71±0.05 Ba	1.01±0.06 Aa	0.63±0.04 Cc	0.78±0.05 Bb	0.94±0.07Ab	0.97±0.09 Ac
	MN	0.74±0.06 Da	0.78±0.04 Db	0.78±0.05 Db	0.87±0.06 Ca	1.00±0.09 Bb	1.35±0.12 Ab
	SN	0.73±0.05 Ea	0.70±0.05 Eb	0.95±0.08 Ca	0.81±0.06 Dab	1.21±0.11 Ba	1.99±0.14 Aa

Note: The different capital letters indicate significant difference between different treatment time under the same treatment ($P<0.05$). The different lowercase letters indicate significant difference between different treatments under the same treatment time ($P<0.05$).

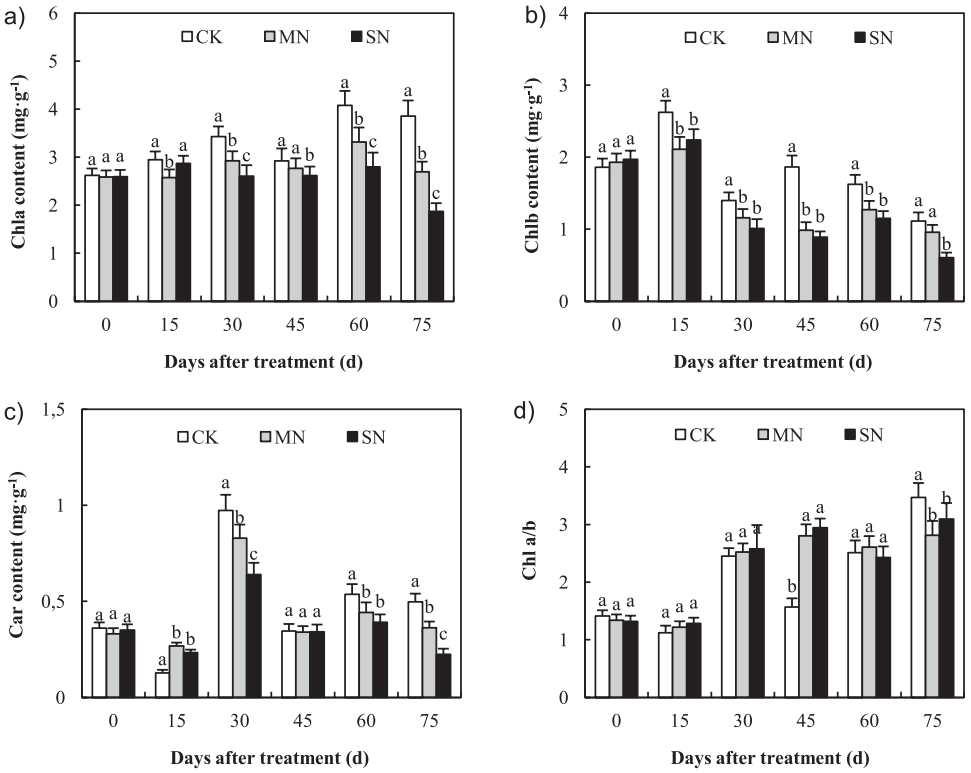

Fig. 1. The photosynthetic pigment contents in leaves of walnut seedlings under different treatments (mean±SE).

Changes of Photosynthetic Pigment Content in Leaves of Walnut Seedlings under Different Treatments

As shown in Fig. 1a), with the extension of treatment time, the change trend of Chl *a* content in the leaves of MN group was basically consistent with that of the CK group, which gradually rose in the early stage and clearly dwindled after 60 d ($P<0.05$), while the Chl *a* content in leaves of SN group had little change in the early stage ($P>0.05$) and was markedly reduced after 60 d ($P<0.05$). With the prolongation of treatment time, the Chl *b* content in leaves of each group generally rose at the beginning, and then was gradually depleted after 15 d (Fig. 1b). The Car content of different groups increased at first and then diminished (Fig. 1c). With the prolongation of treatment time, the Chl *a/b* values of leaves in CK group did not change regularly, while in MN group and SN group, it demonstrated an overall upward trend (Fig. 1d). With the increase of N deficiency level, the contents of Chl *a*, Chl *b*, and Car dwindled conspicuously ($P<0.05$), and the contents of SN group were the lowest. Moreover, the higher the degree of N deficiency, the higher the Chl *a/b* in the early stage, and the Chl *a/b* in SN group became the lowest after 60 d.

Changes in Photosynthetic Parameters of Walnut Seedlings with Different Treatments

As shown in Fig. 2a), at the initial stage of treatment (0-15 d), *Pn* of walnut seedlings in MN group and SN group diminished conspicuously ($P<0.05$), then were gradually enhanced, and declined obviously after 30 d ($P<0.05$). Throughout the experiment, the *Pn* of MN group and SN group was notably lower than that of CK group in general ($P<0.05$). With the prolongation of treatment time, *gs* and *Tr* of walnut seedlings under different treatments generally presented a similar trend, which crept up in the early phase and depressed markedly after 60 d ($P<0.05$) (Fig. 2b, 2c). Under different N deficiency conditions, the *gs* and *Tr* in CK group were generally the highest, followed by MN group, and the lowest in SN group. As shown in Fig. 2d), at the initial stage of treatment (0-15 d), the water use efficiency of each group of walnut seedlings diminished notably ($P<0.05$), then was enhanced by degrees, and the change tended to be gentle after 30 d. Besides, during the period 0-30 d, the water use efficiency was comparable between MN group and CK group, without distinct discrepancy ($P>0.05$), and conspicuously higher than SN group ($P<0.05$). However, after 30 d, compared with CK group, the water use efficiency of SN group was improved.

Changes of Endogenous Hormone Content in Walnut Seedlings under Different Treatments

As shown in Fig. 3a), in the entire process of the experiment, the IAA content of walnut seedlings in the CK group had no obvious change ($P>0.05$). By contrast, IAA content under N deficiency treatment appeared increase tendency, and the growth rate accelerated

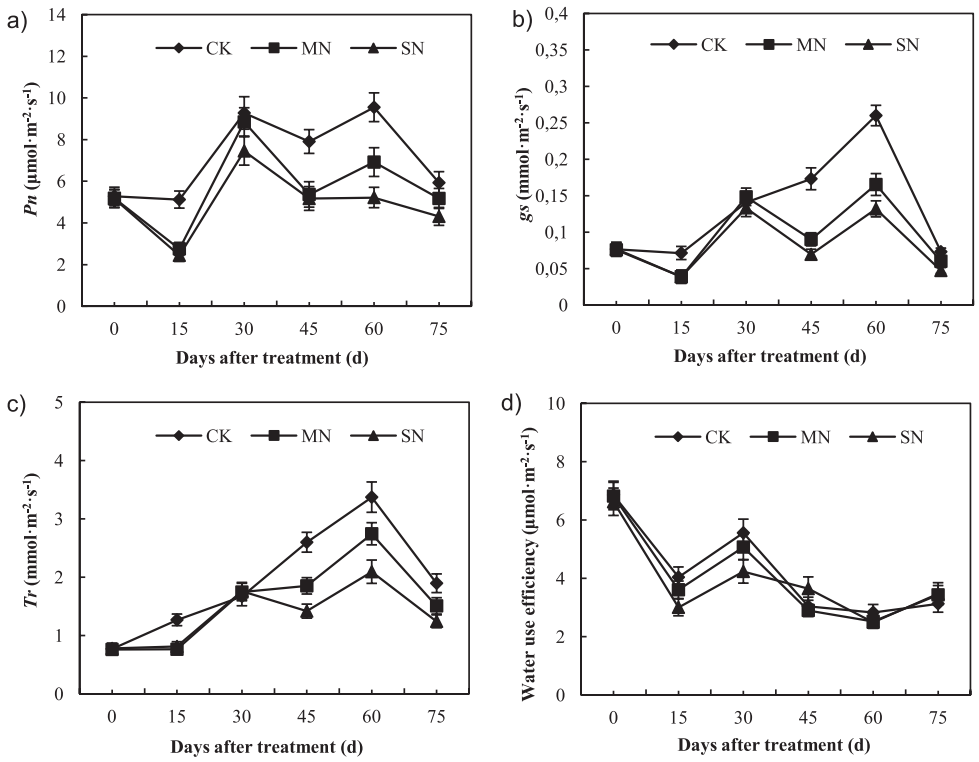

Fig. 2. The photosynthetic parameters of walnut seedlings under different treatments (mean±SE).

noticeably after 45 d. During the experiment, the IAA content in SN group was the highest, followed by MN group and CK group. As shown in Fig. 3b), the CTK content of walnut seedlings under different N deficiency treatments was on an uptrend within 30 d, and a considerable downward trend after 30 d ($P<0.05$). Generally speaking, the CTK content was the highest in SN group, followed by MN group, and that in CK group was the lowest. As shown in Fig. 3c), in the early stage, the ABA content of walnut seedlings in

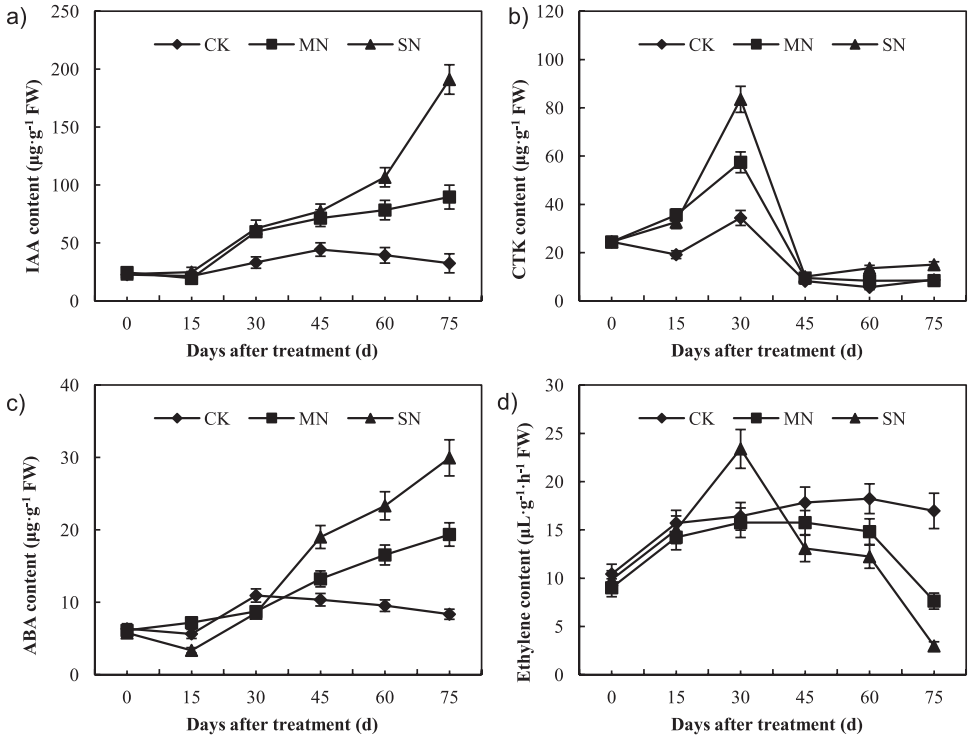

Fig. 3. The endogenous hormone content of walnut seedlings under different treatments (mean±SE).

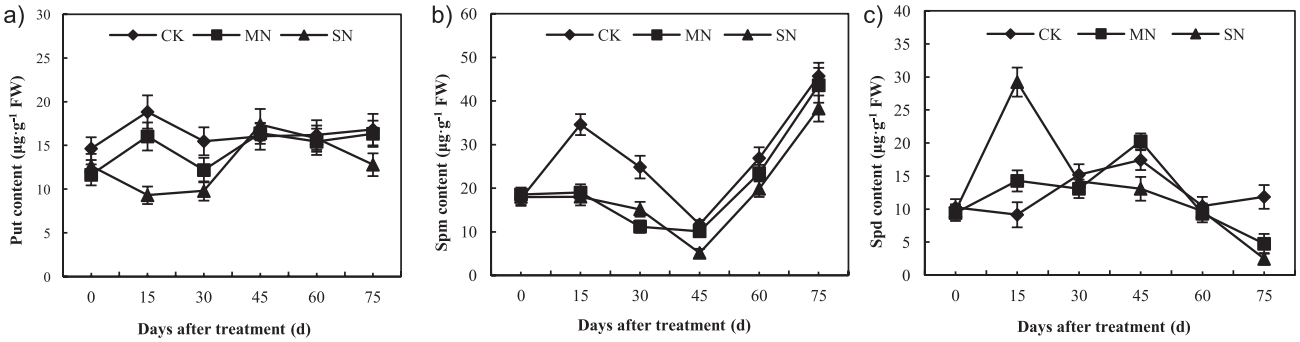

Fig. 4. The polyamine content of walnut seedlings under different treatments (mean±SE).

each group had less distinctive disparity. After 30 d, the ABA content of SN group and MN group had distinct increments compared with CK group, and with the extension of processing time, the difference with the CK group gradually expanded ($P<0.05$). However, the ABA content of CK group had little change as a whole. Under different N deficiency treatments, the ABA content in SN group was generally the highest, followed by MN group, and the lowest in CK group. As shown in Fig. 3D, the ethylene contents of walnut seedlings in the two N-deficiency groups showed an upward trend from 0 to 30 d, and followed by a dramatic decrease after 30 d ($P<0.05$). However, no apparent variations in the ethylene content of CK group were observed throughout the treatment, and the ethylene content of CK group was generally markedly higher than that of MN and SN groups. It was only at 30 d that the ethylene content of SN group was noticeably higher than that of MN group and CK group ($P<0.05$).

Changes of Polyamine Content in Walnut Seedlings under Different Treatments

As shown in Fig. 4a), with the prolongation of treatment time, the variation trend of Put content in walnut seedlings in the MN group was similar to that in the CK group. There was a remarkable upward trend at the early stage (0-15 d) ($P<0.05$), and then gradually stabilized, with no considerable difference ($P>0.05$). However, the Put content of SN group dwindled significantly in the early stage (0-15 d) ($P<0.05$), followed by a substantial increase after 30 d ($P<0.05$). Generally, Put content decreased conspicuously with increasing levels of N deficiency ($P<0.05$) throughout the trial process. As shown in Fig. 4b), with the extension of processing time, the Spm content of walnut seedlings under different treatments dwindled steadily at the beginning, then rose. During the whole treatment process, the Spm content was generally the highest in the CK group, followed by the MN group, and the lowest in the SN group. As shown in Fig. 4c), the Spd content of walnut seedling in the CK group varied little during the whole course of the experiment. Both N deficiency treatment groups showed a trend of increasing Spd content at first and then decreasing Spd content. At the initial stage of treatment (0-15 d), the Spd content was the highest in SN group, followed by that in MN group, and the lowest in CK group, with significant differences among the groups ($P<0.05$). In the middle treatment period (30-45 d), the Spd content in MN group was the highest, followed by CK group, and that in MN group was the lowest, and there was statistical difference among the groups ($P<0.05$). At the later stage of treatment (60-75 d), the Spd content in the CK group was the highest, followed by the MN group, and the lowest in the SN group. And distinct deviation existed among the groups ($P<0.05$).

Changes of Root Activity in the Walnut Seedlings under Different Treatments

As shown in Fig. 5, at the initial stage of treatment (0-30 d), the root activity upsurged conspicuously under different treatments ($P<0.05$). After that, the root activity of CK group tended to stabilize without statistical difference ($P>0.05$). The root activity of MN group consistently remained at a high level, and continued to increase remarkably at the late stage of treatment (60-75 d), while the root activity of SN group

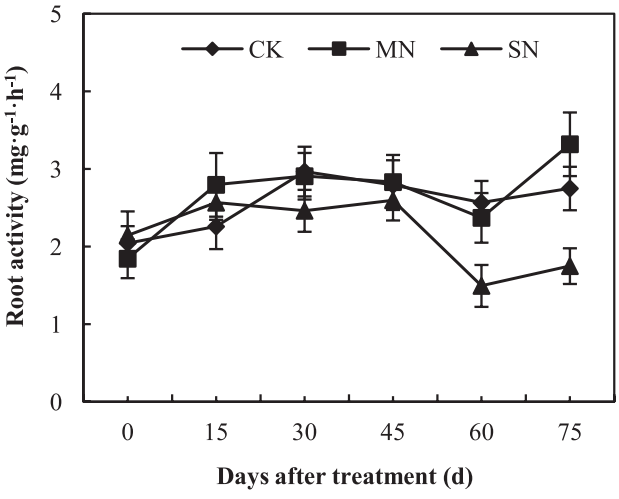

Fig. 5. The root activity of the walnut seedlings under different treatments (Mean±SE).

decreased remarkably after 45 d ($P<0.05$). On the whole, the root activity of the MN group was generally the highest, followed by the CK group, and the SN group was the lowest.

Conclusions

Chlorophyll plays a major role in absorbing and transmitting light energy during photosynthesis. The content of chlorophyll directly determines the rate of photosynthesis in plants. N is the primary element for chlorophyll synthesis. N deficiency will lead to a striking reduction of chlorophyll content and yellowing of leaves [19-20]. The results showed that the contents of Chl *a*, Chl *b*, and Car in walnut seedlings under N deficiency were distinctly lowered compared with CK group. Such a reduction was even more pronounced with the increase in N deficiency stress level. In addition, long-term N deficiency stress resulted in significantly lower contents of Chl *a* and Chl *b* than those before treatment, indicating that N deficiency led to a shortage of material sources for chlorophyll synthesis. Long-term N deficiency stress could also destroy the photosynthetic mechanism in chloroplasts, thereby inhibiting the synthesis of chlorophyll. The factors that lead to the reduction of photosynthesis rate can be divided into stomatal limitation and non-stomatal limitation. The first case means that the reduction of *gs* will lower the CO_2 supply; the second one refers to a reduction in the photosynthetic capacity of mesophyll cells, which eventuates in the decrease of CO_2 utilization capacity of mesophyll cells [18, 21]. In this study, compared with CK group, the *gs* of walnut seedlings was severely depleted under N-deficiency stress, especially under severe N-deficiency. These findings indicated that under N deficiency stress, walnut seedlings were subjected to the dual influence of stomatal-related and non-stomatal-related limiting factors. Furthermore, N is one of the primary constituent elements of key enzymes in photosynthesis. N deficiency could lead to the inability to synthesize such enzymes [22] and hence a dramatic reduction in the photosynthesis rate of walnut seedlings. As photosynthesis was weakened in walnut seedlings under N deficiency stress, the products of photosynthesis declined, and so did the biomasses of aboveground and underground parts. As the level of N deficiency increased, the seedling growth became more sluggish.

Transpiration can promote the absorption and transportation of water by plants, during which mineral nutrients are transported from the roots to the aboveground parts to meet the needs of plant growth [23-24]. Numerous studies have shown that N deficiency could affect transpiration [21, 25-26]. We also found that the transpiration rate (*Tr*) of walnut seedlings decreased more remarkably with increasing N deficiency. According to Radin [27], under N deficiency stress, the turgor pressure of root cells and the elasticity coefficient of cell walls in the cotton seedlings were enhanced. As a result, water exchange slowed down, and water transportation abated. It is believed that the biophysical properties of the endodermal cell membrane in the roots restrict the flow of water to the steles, thereby affecting transpiration. We observed a dramatic reduction in root biomass of walnut seedlings under N deficiency stress. Apparently, N deficiency stress greatly influenced the absorption capacity of walnut seedlings to water and mineral nutrients. With the extension of stress time, the root: shoot ratio of walnut seedlings rose significantly. At the end of the experiment, the root: shoot ratio of the seedlings under severe N deficiency was 105.1% higher than that of the CK group. These seedlings exerted an autoregulatory effect under N deficiency stress, improving absorptive capacity by increasing root capacity. Moreover, the root activity detected in this study was expressed in terms of succinate dehydrogenase content, which in turn was closely related to respiration [15]. Severe N deficiency stress caused a dramatic reduction in root vigor of walnut seedlings. In contrast, the root vigor of seedlings under moderate N deficiency was higher than that in CK group. Studies on *Alnus* [28] and *Populus* [29] showed that low concentrations of N were conducive to increasing root respiration rate. Thus, under the condition of moderate N deficiency, the respiration of walnut seedlings could be enhanced, which further accelerated the circulation of material and energy, hence improving the absorptive capacity.

Endogenous hormones are crucial for plant resistance. Any environmental factor at stress level can cause changes in the content and balance of endogenous hormones. Different endogenous hormones interact with each other in a very complicated manner in plants to cope with environmental changes [30-31]. During plant growth, IAA can promote cell elongation and accelerate nuclear division [32], while CTK plays a role in cytoplasmic division, therefore such an effect can only be manifested in the presence of IAA [33-34]. In the present study, the contents of IAA and CTK increased significantly in walnut seedlings under N deficiency stress, which was probably to synergistically accelerate cell division, maintain vigorous growth of plants and cope with adversity stress. In the meantime, the increase of CTK content is favorable to improving the N utilization efficiency [35] and the tolerance of walnut seedlings to N deficiency. With the prolongation of treatment time, IAA content of walnut seedlings under N deficiency stress increased continuously, while the CTK content was depleted distinctly in the later period. Possibly because CTK is an N-containing heterocyclic compound, and N is the main constituent element, at the later stage of the experiment, N deficiency hindered the synthesis of CTK in walnut seedlings, causing a sharp reduction in CTK content. CTK can facilitate the migration of IAA in the polar regions. As the decrease of CTK content, the transport of IAA was affected, and IAA accumulated abundantly

in the leaves of walnut seedlings. Consequently, normal protein and nucleic acid metabolism was affected and plant growth was inhibited [23]. Under the condition of short-term N deficiency, the increase of IAA and CTK content was conducive to accelerate cell division, improve N utilization efficiency, and alleviate stress. However, long-term N deficiency would disrupt the balance between IAA and CTK content, which was unfavorable for plant growth.

Chapin et al. [36] showed that N deficiency caused an apparent increase in ABA content in leaves, which was considered to be a normal plant response to stress. That is, plants produce ABA to promote stomatal closure and dormancy as a way of self-protection. Under N deficiency stress, we observed a remarkable increase in ABA content in walnut seedlings in the present study, and such an increase became more conspicuous with increasing levels of N deficiency. As a consequence, under severe N deficiency conditions, g*s* abated remarkably, leading to a marked weakening of photosynthesis. CTK could inhibit ABA synthesis and thus has an anti-aging effect [37]. We also found that the CTK content of walnut seedlings under N deficiency was enhanced,from 0 to 30 d, and started to decrease significantly after 30 d, while the ABA content presented the opposite trend, first decreased and then rose. This result indicated that ABA played a more significant role at the late stage of N deficiency, resulting in more retarded growth of walnut seedlings. Several studies have shown that ABA can stimulate ethylene synthesis, which in turn promotes cellular senescence and cell wall decomposition [38-39]. Lee et al. [40] investigated the response of rice seedlings to different N levels. They found that as N levels increased, the methionine content of rice seedlings was depleted, while the ethylene content was raised. They concluded that N deficiency stimulated the conversion from 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene. According to this study, the ethylene content of walnut seedlings under N deficiency stress increased at the beginning of the experiment compared with the CK group. However, the ethylene content lessened dramatically after 30 d, which was significantly lower than that in the CK group. The variation trend of ethylene content was opposite to that of ABA content. The reason might be that the initial precursor for ethylene synthesis was methionine. However, there was an N deficiency at the late stage, which inhibited the synthesis of precursors and relevant enzymes and further affected ethylene synthesis. Thus, under N deficiency, high content of ABA and ethylene could promote the dormancy of walnut seedlings and alleviate the injury. However, chronic N deficiency could still result in an imbalance of endogenous hormones. Dormancy induced by N deficiency under normal conditions may severely inhibit plant growth or even cause senescence of the entire plant.

Many studies indicated that under the stress of salinity, drought, cold and heat injury, heavy metals, and mechanical injury, plants could produce a series of signals to regulate the synthesis, decomposition, and metabolism of polyamines, affecting homeostasis. Richards et al. [41] were the first to report the intensive accumulation of Put in barley leaves under potassium deficient stress. Subsequent experiments also demonstrated that potassium deficiency could cause an increase in Put content. Other types of nutrient stress, such as magnesium deficiency, excess ammonia, and excess calcium, may lead to increased Put content and arginine decarboxylation (ADC) activity in plants [42-43]. According to another study, the polyamine content in plants abated under adversity stress. For example, the polyamine content of tomatoes was gradually lowered under salinity stress with the prolongation of stress time [44]; the contents of Put, Spd, and Spm in the fruits of muskmelons lessened considerably under low temperature at night [45]. We also found that under N deficiency stress, the contents of Put, Spd, and Spm of walnut seedlings were generally distinctly lower than those of the CK group. There was no polyamine accumulation under N deficiency. Arginine and ornithine were precursors for polyamine synthesis in plants, and Put was synthesized in the presence of a series of key enzymes, such as ADC and ornithine decarboxylase (ODC). Then aminopropyl was added continuously under the action of specific enzymes to form Spd and Spm. Additionally, Spd and Spm were also derived from methionine by losing a carboxyl group [46]. As shown above, the synthesis of several polyamines and relevant enzymes requires a large amount of N supply. Among them, ADCs in plants are very sensitive to external stimuli and stresses. Watson et al. [47] identified two ADC-encoding genes in *Arabidopsis thaliana*, namely ADC1 and ADC2. ADC1 is expressed in all plant tissues, whereas ADC2 expression is associated with some adversity stress, such as drought and mechanical injury [48-49]. This study concluded that N deficiency stress might fail to induce ADC2 gene expression in walnut seedlings. In addition, the content of precursors and key enzymes, involved in polyamine synthesis in walnut seedlings, abated due to the N deficiency stress. The combination of these factors induced a reduction in the content of several polyamines. Other studies have shown that polyamines can stimulate plant growth and delay senescence [50]. Therefore, external use of polyamines might promote the growth and tolerance of walnut seedlings under N deficiency, and improve N use efficiency, but further research is needed.

To conclude, under N deficiency stress, large quantities of ABA were synthesized in walnut seedlings, which induced stomatal closure and weakened photosynthesis and transpiration. ABA might serve as a signal substance to regulate the content of such endogenous hormones as IAA, CTK, and ethylene, which in turn accelerated cell division, improved N utilization and alleviated stress. Under short-term N deficiency stress, more biomasses were allocated to

the roots of walnut seedlings to enhance respiration and material-energy cycle, and ultimately improve the nutrient absorption capacity. However, long-term N deficiency could induce disordered synthesis of endogenous hormones and polyamines, weakened photosynthesis, and retarded root growth. As a result, the growth of the entire plant was affected, and plant senescence might be accelerated.

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Conflict of Interest

The authors declare no conflict of interest.

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