THE INTERACTIVE EFFECTS OF N SUPPLY AND TEMPERATURE ON PHOTOSYNTHESIS AND TRANSCRIPTION EXPRESSION OF RUBISCO AND ITS ACTIVASE GENES IN THE SEEDLINGS OF CAMELLIA OLEIFERA

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Abstract

In this study, we assessed the interactive effects of nitrogen (N) gradients under different temperatures on the net photosynthetic rate (Pn), intercellular CO₂ concentration (Ci), stomatal conductance (gs), transpiration rate (E) and in vivo velocity of Rubisco carboxylation (Vc) for 'Xianglin 14' seedlings of Camellia oleifera. We found that Pn was higher at the moderate N concentration than those at lower and higher N concentrations at 15°C and 20°C, and gs and E exhibited the same trend as Pn with exception of Ci (due to the some reverse recorders), suggesting that the moderate N supply was more favorable for photosynthesis under 20°C. Furthermore, transcript patterns of Co-rbcL, Co-rbcS and Co-RCA presented similar tendencies as these photosynthetic parameters, while the redundant N down-regulated transcripts of these genes. In addition, these photosynthetic parameters and their corresponding molecular evidences displayed significant correlations, indicating that the transcript level of Co-RCA was a more optimal molecular marker to evaluate regulatory effects of N and temperature on seedlings than those of Co-rbcS and Co-rbcL. Collectively, our study quantified the relationships among temperature, N supply, photosynthetic parameters and the transcriptions of Rubisco related genes, and affirmed that physiological and molecular assays were sufficiently sensitive to resolve the interactive effects of N supply and temperature. Ultimately this allows optimization of management practices to improve the photosynthetic efficiency.

Key words: Camellia oleifera, Nitrogen, Temperature, Effect, Photosynthesis, Rubisco, Rubisco activase, Transcription.

Introduction

Nitrogen (N), a constituent of proteins, amino acids, RNA and DNA, is an essential macronutrient for plant physiology, and its greatest impact on plant nutrition may be photosynthesis. And its available extent may affect photosynthetic parameters of the net photosynthetic rate (Pn), transpiration rate (E), intercellular CO₂ concentration (Ci), stomatal conductance (gs), and in vivo velocity of Rubisco carboxylation (Vc), etc (Reddy et al., 1996; Clearwater & Meinzer 2001; Good et al., 2004; Correia et al., 2005; Nicodemus et al., 2008). Appropriate nitrogen addition can improve the leaf photosynthetic ability (e.g. Pn, and Vc) of the plants, such as Eucalyptus grandis, Juglans nigra and Triticum aestivum. While excessive nitrogen adding process, make leaf photosynthetic capacity decreased (Clearwater & Meinzer 2001; Nicodemus et al., 2008; Shabbir et al., 2015). Moreover there exists a highly positive correlation between photosynthetic capacity and N supply (Evans, 1989; Ripullone et al., 2003; Wang et al., 2012; Yan et al., 2014). Apart from nitrogen, temperature also significantly affects photosynthetic parameters. And lower temperature will lead to photosynthetic capacity decrease. While related high temperature could increase the photosynthetic capacities (Lin et al., 2012; Yamori et al., 2014). In addition, Fc also increase with the increase of temperature and N supply (Pons, 2012; Yan et al., 2014).

Besides physiological effects, N and temperature exert great influence on the transcripts of the Rubisco-related genes, and thus largely affects the photosynthesis of higher plants. To some extent, N and temperature affect photosynthetic characteristics through activating Rubisco and regulating the expression of the Rubisco related genes (Suzuki et al., 2007; Sharwood et al., 2008). Rubisco is composed of 8 small subunits encoded by a small nuclear multigene (rbcS) family, and 8 large subunits encoded by a single gene (rbcL) in the multi-copy chloroplast genome (Schneider et al., 1992). For instance, the activated Rubisco by nitrogen nutrition is associated with the increases of rbcL, rbcS mRNA to a similar extent (Imai et al., 2005; Imai et al., 2008; Suzuki et al., 2007); the relative transcript abundances of rbcL and rbcS affect photosynthetic properties, photosynthetic efficiency or capacity, and their total RNA levels exhibit qualitatively similar increase patterns with N supply increase (Flood et al., 2011; Imai et al., 2005; Sharwood et al., 2008). While RCA (Rubisco activase) catalyzes the activation of Rubisco and plays a crucial role in photosynthesis, its down-regulation expression might cause the photosynthetic capacities dramatically to decline. Its transcription expression pattern tends to the qualitatively similar patterns as that of rbcS and rbcL mRNAs (Zielinski et al., 1989; Eckardt et al., 1997; He et al., 1997; Yin et al., 2010).

Even though N and temperature taken together or separately exert a great influences on photosynthesis as well as the gene transcript expressions of Rubisco-related on other higher plants, the studies of their combination effects are limited. Moreover, Camellia oleifera, the most important edible oil trees of China, producing a high-quality vegetable oil with the higher edible value as well as the better medicinal value has a lower seed-oil yield of per unit area (Lee & Yen, 2006; Zhuang, 2008; Chen et al., 2015). So, to improve photosynthetic capacity, and in turn the seed-oil yield is perhaps an ideal approach (Chen et al., 2015). To date, however, little has focused on the
combination effects of N and temperature on photosynthesis of C. oleifera. Thus, in this study, we investigated interactive effects of N addition on the photosynthetic parameters of C. oleifera seedlings under different temperatures, and aimed (1) to clarify how N affects photosynthetic characteristics of these seedlings under different temperatures (2) to determine an optimal combination of N supply and temperature for seedlings so as to improve photosynthetic capacities (3) to demonstrate the transcript patterns of the Rubisco related genes, and expect to find a molecular marker to assess regulatory effects of N and temperature on C. oleifera seedlings.

Materials and Methods

Plant materials and growth conditions: The sand-stored seeds of ‘Xianglin 14’ were placed in the Petri dish covered with wet filter paper for germination, Four weeks later, the germinated seeds were transferred to 6×6 cm plastic pots with vermiculite, and cultured in the climate cabinets. The photoperiod was 14 h light/10 h dark (6:00-20:00) with light intensity 50 μmol photons m−2 s−1, day/night temperature of 25/20 °C and 60% relative humidity. Two groups of seedlings were irrigated with a solution containing NH4NO3 at the following seven different N concentrations (mM): the N control (N-C, without N sources), 0.5 (0.25 mM NH4NO3), 2 (1 mM NH4NO3), 8 (4.0 mM NH4NO3), 10 (5.0 mM NH4NO3), 20 (10.0 mM NH4NO3) and 50 (25.0 mM NH4NO3), respectively along with a balanced mixture of the other major nutrients 3 mM KH2PO4, 1 mM MgSO4.7H2O, 3 mM CaCl2, 25 μM H3BO3, 2 μM MnSO4.5H2O, 2 μM ZnSO4.7H2O, 0.5 μM CuSO4.5H2O, 0.5 μM Na2MoO4·H2O, and 20 μM Fe-EDTA(Suzuki et al., 2007; Sugiura et al., 2012). Two groups of N-treated seedlings were placed in the climate cabinets of 15 and 20°C for a month, respectively under the photoperiod described above. All analyses were conducted the fully expanded leaves.

Determination of the photosynthetic parameters and characteristics: LI-6400 photosynthesis analyzer (LI-Cor, Lincoln, NE, USA) was used to measure the net photosynthetic rate (Pn), stomatal conductance (gs), intercellular concentration CO2 [CO2] (Ci), and transpiration rate (E) of the fully expanded leaves of seedlings. The [CO2] in the leaf chamber was controlled by the LI-Cor CO2 injection system, and the built-in LED lamp (red/blue) supplied the irradiance. The values of the A/Ci (leaf net CO2 assimilation rate versus intercellular concentration CO2 [CO2]) response curves were used to calculate in vivo velocity of Rubisco carboxylation (Vc) under [CO2] of 50, 100, 150, 200 and 250 μmol mol−1 (Farquhar et al., 1980).

The preparation of total RNA and the first strand cDNA synthesis: Total RNA was extracted from the photosynthetic determined leaves, ground in liquid nitrogen and lysed using 600 μl 3×CTAB with 1% 2-mercaptoethanol, followed by e.Z.N.A.TM® Plant RNA Kit Reagent (OMEGA) according to the manufacturer’s instructions. RNase-free DNase (OMEGA) was used to remove any remaining contaminating DNA from the total RNA extractions. An aliquot of each sample was run on an agarose gel. The concentration and purity of each RNA sample were checked with Nano-drop 2000 (Thermo Scientific). Total RNA (500 ng) was used to generate the corresponding single-stranded cDNAs using an anchored oligo (dT)18 primer and M-MLV reverse transcriptase in the presence of an RNase inhibitor (TaKaRa) in a 20-μl volume.

Transcript abundance analysis of Rubisco related genes by qPCR: Aliquots of the single-stranded cDNA were used as templates for qPCR analysis on CFX96 (Bio-Rad). PCR amplification was performed in a total volume of 20 μl. The reaction volumes contained 10μl SYBR Premix Ex Taq (2×) (Tli RNase H Plus) (TaKaRa), 0.6 μl of each primer (10 μM) (Table 1), and 2.0μl of 10-fold cDNA dilutions as PCR templates, and RNase free water to bring the volume up to 20 μl. Cycling consisted of 94°C for 30 s, followed by 40 cycles of 94°C for 15 s, 54°C to 59°C (according to Table 1) for 30 s, 72°C 30 s. Expression of Co-GAPDH was examined as an internal control of Co-rbcL and Co-rbcS; and Co-GAPDH and Co-Actin were used as the reference genes of Co-RCA. The sizes of amplicons were 194, 165, 107, 185 and 111 bp, respectively (Table 1) (Wang et al., 2012). Data analysis was performed using Bio-Rad CFX 2.0 data analysis software. All samples were replicated 3 times.

Statistical analysis: The effects of N and temperature on photosynthetic parameters and the transcription of the Rubisco related genes Co-rbcL, Co-rbcS and Co-RCA were analyzed with statistical analysis software SPSS17.0. The results were presented as the means of three independent experiments. Pearson correlation coefficients were analyzed by test of significance for two-tailed at the level of p<0.01.

Results

Feature and variation of leaf photosynthesis: To assess how temperature and N supply were responsible for variations of the photosynthetic capacities, we transferred these seedlings treated with the N gradient (0, 0.5, 2.0, 8.0, 10.0, 20.0 and 50.0 mM) supply to 15 and 20°C temperature conditions, respectively, and found that Pns were proportionally increased from the N control to N 10.0 mM level supply by about 46.9% and 48.8% at 15 and 20°C respectively (Fig. 1a), then declined from the moderate (10.0 mM) to higher N level (50.0 mM) with ratios approximately 29.0% and 26.5% under 15 and 20°C, respectively. This indicates that the moderate N level was the most suitable for photosynthesis of C. oleifera seedling. Moreover, the average Pns of N supply concentrations were correspondingly increased by about 36.3% for 15°C vs 20°C (calculated from Fig. 1a), suggesting that the effect of N and temperature to Pn were dependent on temperature and the N supply. Moreover, alterations of gs and E were positively correlated with Pn for all N treatments under two temperature conditions as observed from Fig. 1b, Fig. 1d and Table 2. While Ci presented the almost reverse trends of ‘high-low-high’ in comparison to those of Pn, gs and E, of which values under the moderate N (8.0 mM) were lower than those under higher N supply (50.0 mM) at 20°C (Fig. 1c). Moreover, Ci was negative correlated with gs and E (r = -0.828, -0.796, p<0.01). In addition, gs was significantly positive correlated with E (r = 0.984, p<0.01) (Table 2).
The transcript abundances of transcript amounts for the two genes of the N-treated 3). The above results, together with the maximal of r significantly correlated (r = 0.748, **p < 0.01 level), and with Vc at **p < 0.01 level from Table 3. The transcript expression pattern of Co-RCA was almost identical to those of Co-rbcL and Co-rbcS, and also presented trends of ‘low-high-low’ with N supply and temperature increase (Fig. 4c). The transcript abundances of Co-RCA from the N control to moderate N (8.0 mM, 10 mM) was significantly altered at 15 and 20°C, respectively. And the transcript of Co-RCA largely declined from the moderate (8.0 mM, 10 mM)) and higher N (50.0 mM) under both 15 and 20°C, respectively. The average amount of Co-RCA was greatly higher under 20°C than those under 15°C (Fig. 3c). The result suggested that the suitable N enhanced the transcript abundance of Co-RCA, while the excessive N seriously inhibited its transcript level under each temperature, and that 20°C was more favorable for the transcript expression of Co-RCA. Moreover, the Pearson correlation coefficient of Co-RCA vs Co-rbcS (0.747) was higher than that (0.608) of Co-RCA vs Co-rbcL at p < 0.01 level from Table 3. Notably, Co-RCA had significant correlations with Pn (r = 0.822), and with Vc (r = 0.701) at p < 0.01 level. Collectively, our finding was similar to the previous result that transcript expression of RCA tended to the qualitatively similar patterns as those of rbcS and rbcL mRNAs (Zielinski et al., 1989; He et al., 1997; Eckardt et al., 1997; Yin et al., 2010), and supports the fact that RCA plays a crucial role in photosynthesis (Yin et al., 2010).

Table 1. The primer sequences for qPCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
<th>AT (°C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbcL</td>
<td>TGTAATACAGTTCCGGGGAG</td>
<td>TTCATACCTCACAGCGCA</td>
<td>59</td>
<td>194</td>
</tr>
<tr>
<td>rbcS</td>
<td>TGGCGGTAGTCACTGACAGACTG</td>
<td>CAGGGGATGAAGACTGAGCGA</td>
<td>59</td>
<td>165</td>
</tr>
<tr>
<td>RCA</td>
<td>ATTCGTCATCGTGCTTATGGA</td>
<td>ACATCCTCATCGGGGCA</td>
<td>58</td>
<td>107</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAAGGGTGGTGCCAAAGAAG</td>
<td>GACCTCCAACAAATGGCGAAC</td>
<td>58</td>
<td>185</td>
</tr>
<tr>
<td>actin</td>
<td>ATGTACTACGATAGAAGAAT</td>
<td>ATGTACGACTGAAAGAAGA</td>
<td>54</td>
<td>111</td>
</tr>
</tbody>
</table>

AT: Annealing temperature (°C)

**Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level**

The interactive effects of N supply and temperature on photosynthesis parameters C. oleifera seedlings.

<table>
<thead>
<tr>
<th>Vc</th>
<th>Pn</th>
<th>Gs</th>
<th>Ci</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vc</td>
<td>1</td>
<td>0.748(**)</td>
<td>-0.708(**)</td>
<td>0.702(**)</td>
</tr>
<tr>
<td>Pn</td>
<td>1</td>
<td>0.813(**)</td>
<td>-0.755(**)</td>
<td>0.834(**)</td>
</tr>
<tr>
<td>Gs</td>
<td>1</td>
<td>-0.828(**)</td>
<td>0.984(**)</td>
<td></td>
</tr>
<tr>
<td>Ci</td>
<td>1</td>
<td>-0.796(**)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level**

Discussion

Effects of temperature and nitrogen on photosynthesis parameters: Our study has demonstrated that variations of photosynthetic parameters in response to N and temperature. From Fig. 1 and Fig. 2, we found that leaf photosynthetic characteristics were associated with N level, and the moderate N level was favorable for the performances of photosynthetic parameters, and confirmed a causual relationship between N nutrition and photosynthesis as described by Reddy et al. (1996) and Correia et al. (2005). Of which Pn, gs and E were positively correlated with N increase at certain temperatures (Reddy et al., 1996).
Fig. 1. The determination of photosynthetic parameters of *C. oleifera* seedlings.
The x-axis represents N concentration of N control (CK), 0.5, 2.0, 8.0, 10, 20, and 50 mM. The two columns represent the corresponding determination amount at 15°C and 20°C, respectively. Fig. 1a, *Pn* (the net photosynthetic rate, μmol CO₂ m⁻²s⁻¹); Fig. 1b, *gs* (stomatal conductance, molH₂O m⁻²s⁻¹); Fig. 1c, *Ci* (intercellular concentration of CO₂, μmol mol⁻¹); Fig. 1d, *E* (transpiration rate, mmol m⁻²s⁻¹).

Fig. 2. The determination of velocity of the Rubisco carboxylation (*Vc*) of *C. oleifera* seedlings.

Fig. 3. The extracted RNAs of *C. oleifera* seedlings under N concentration of the control (CK), 0.5, 2.0, 8.0, 10.0, 20.0, and 50.0 mM at 15°C and 20°C, respectively. A, the RNAs of leaves at 15°C; B, the RNAs of leaves at 20°C.
The determination of the optimal combination of N supply and temperature through response of photosynthesis response: By comparing photosynthetic performances of C. oleifera seedlings under 20°C with those under 15°C, we found that 20°C was more favorable temperature for C. oleifera seedlings photosynthesis than 15°C. Although Pns of seedlings under 20°C were slightly lower than those under 25°C, the values of Vcs for seedlings treated with 0.50 and 8.0 mM N concentration at 20°C were slightly higher than those under 25°C (0.0276 vs 0.0252; 0.0286 vs 0.0261, Table 4), suggesting that 20°C to 25°C was more optimal temperature range for photosynthesis of C. oleifera seedlings. Furthermore, Pn, Vc and transcript levels of Rubisco related genes were significant higher at N 8.0 to 10.0 mM than those at the lower and higher N level at the two temperatures (Figs. 1 and 2). Excessive N (50.0 mM) seriously inhibited the growth of seedlings, and even caused leaves to dry and die, and eventually plant death. Collectively, we determined the favorable combination of N and temperature might be 8.0 mM to 10. mM and 20°C to 25°C, respectively. In addition, we also try to ascertain N supply and temperature which plays the important roles in photosynthesis of C. oleifera seedlings. From Fig. 1, we found that changes of N control in some extent reflected the direct effects of temperature, while other N treatments reflects the indirect effects under the two temperatures. Together with the altered amounts of Pn and Vc shown in Figs. 1 and 2 and Table 4, we considered that temperature might play the relative larger roles in photosynthesis at suitable N level concentrations, our results is consistent with results of those pervious studies (Clearwater & Meinzer, 2001; Nicodemus et al., 2008; Pons, 2012; Yan et al., 2014).

Molecular marker responding to N and temperature of seedlings: We compared the relationships of transcript levels of three Rubisco-related genes of C. oleifera seedlings affected by N level and temperature (Fig. 4). From Fig. 4a and 4b, and Table 3, we found the Co-rbcS and Co-rbcL mRNA levels were highly positively correlated (Suzuki et al., 2001; Suzuki et al., 2007; Suzuki et al., 2009), and supported the result of Ogawa et al. (2012) and Suzuki & Makino (2012) that the total rbcS mRNA level directly or indirectly affects the transcript level of rbcL, which strictly control the synthesis of Rubisco and photosynthetic capacity while transcript expression of Co-RCA tended to display the qualitatively similar patterns as those of rbcS and rbcL mRNAs (Zielinski et al., 1989; Eckardt et al., 1997; He et al., 1997; Yin et al., 2010). Pearson correlation coefficients among transcript levels of Co-rbcL, Co-rbcS, Co-RCA, Pn and Vc were analyzed (Fig. 4, Table 3), and the transcript level of Co-RCA shared a close relationship with Pn (r = 0.822), and with Vc (r = 0.701) at p<0.01 , which were slight higher than those of Co-rbcS and Co-rbcL. Thus, we believe that Co-RCA might be the relative more optimal marker for assessing regulatory effects of N and temperature on seedlings because of its crucial role and regulatory effects of RCA on photosynthetic parameters as described by Yin et al. (2010).

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Fig. 4 The comparison of transcript levels for Rubisco and Rubisco activase (RCA) genes under different temperature and nitrogen concentration. Fig. 4a, Co-rbcL; Fig. 4b, Co-rbcS; Fig. 4c, Co-RCA. The x-axis represents N concentration of N-control, 0.5, 2.0, 8.0, 10, 20 and 50 mM, two columns represent the normalized fold expression of these genes at 15°C and 20°C, respectively.
Table 3. The Pearson correlation among \( P_n \), \( V_c \), and the transcript levels of the Rubisco related genes of \( C. oleifera \) seedlings.

<table>
<thead>
<tr>
<th></th>
<th>( P_n )</th>
<th>( V_c )</th>
<th>Co-rbcL</th>
<th>Co-rbcS</th>
<th>Co-RCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P_n )</td>
<td>1</td>
<td>0.748(**)</td>
<td>0.554(**)</td>
<td>0.702(**)</td>
<td>0.822(**)</td>
</tr>
<tr>
<td>( V_c )</td>
<td>1</td>
<td>0.489(*)</td>
<td>0.606(**)</td>
<td>0.701(**)</td>
<td>0.608(**)</td>
</tr>
<tr>
<td>Co-rbcL</td>
<td>1</td>
<td>0.811(**)</td>
<td>0.747(**)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-rbcS</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level.

Table 4 Comparison between \( P_n \) and \( V_c \) of \( C. oleifera \) seedlings treated with N gradient concentrations under 20°C and 25°C.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>N concentration gradient</th>
<th>( P_n )</th>
<th>( V_c )</th>
<th>Temperature</th>
<th>N concentration gradient</th>
<th>( P_n )</th>
<th>( V_c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C</td>
<td>N-CK</td>
<td>1.96</td>
<td>0.0233</td>
<td>25°C</td>
<td>N-CK</td>
<td>3.13</td>
<td>0.0289</td>
</tr>
<tr>
<td></td>
<td>N-0.5 mM</td>
<td>2.13</td>
<td>0.0265</td>
<td></td>
<td>N-0.5 mM</td>
<td>3.24</td>
<td>0.0252</td>
</tr>
<tr>
<td></td>
<td>N-2.0 mM</td>
<td>2.45</td>
<td>0.0276</td>
<td></td>
<td>N-2.0 mM</td>
<td>3.58</td>
<td>0.0288</td>
</tr>
<tr>
<td></td>
<td>N-8.0 mM</td>
<td>3.34</td>
<td>0.0286</td>
<td></td>
<td>N-8.0 mM</td>
<td>3.73</td>
<td>0.0261</td>
</tr>
<tr>
<td></td>
<td>N-10.0 mM</td>
<td>3.85</td>
<td>0.0298</td>
<td></td>
<td>N-10.0 mM</td>
<td>4.86</td>
<td>0.0274</td>
</tr>
<tr>
<td></td>
<td>N-20.0 mM</td>
<td>3.67</td>
<td>0.0167</td>
<td></td>
<td>N-20.0 mM</td>
<td>4.06</td>
<td>0.0235</td>
</tr>
<tr>
<td></td>
<td>N-50.0 mM</td>
<td>1.96</td>
<td>0.0121</td>
<td></td>
<td>N-50.0 mM</td>
<td>3.31</td>
<td>0.0137</td>
</tr>
<tr>
<td>Mean</td>
<td>2.77</td>
<td>0.0235</td>
<td></td>
<td>Mean</td>
<td>3.70</td>
<td>0.0248</td>
<td></td>
</tr>
</tbody>
</table>

In summary, our result affirmed that physiological and molecular assays were sufficiently sensitive to resolve the interactive effects of N level and temperature on \( C. oleifera \) seedlings, and that these effects translate to photosynthetic performances, and thus may be helpful to enhance photosynthetic capacities by increasing efficiency of N utilization and by choosing optimal temperature. Furthermore, our study has provided useful rapid screening tools in conjunction with a multi-level approach to the cultivation and management in the seedling stage, and was ultimately beneficial to determine the appropriate N level and temperature, so as to optimize management practices to improve photosynthetic efficiency of seedlings.

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