Application of Optimal Combined Microfertilizers of Boron, Molybdenum, and Copper Improves Root Tuber Yield Trait and Photosynthetic Response Characteristics in *Pseudostellaria heterophylla*

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Abstract. In the actual cultivation process, blind fertilizer application was widespread, resulting in a serious decline in the yield of *Pseudostellaria heterophylla*. We used the 3414 fertilizer experiment design to study the effects of combined Boron (B), Molybdenum (Mo), and Copper (Cu) on the growth indexes, diurnal changes of photosynthesis, and rapid fluorescence induction dynamics in *P. heterophylla*. Our results show that the optimal combination of B, Mo, and Cu simultaneously promoted the growth of underground and aboveground parts, and significantly improved the quality of single root tuber and yield per unit area. The best combination was treatment 9 (T9 = B, 1 g/L; Mo, 0.08 g/L; Cu, 0.05 g/L), and resulted in a 35.1% increase in yield per unit area compared with the control group (T1). Although the optimal combined application of microfertilizers did not change the bimodal trend of diurnal variation of photosynthesis, it effectively increased the daily average, peak, and valley values of the photosynthetic rate by alleviating the nonstomatal limitation and the photosynthetic midday depression. *Pseudostellaria heterophylla* leaves showed greater photochemical activity and less photoinhibition of photosystem II in T9. Major effects were that it helped protect the activity of the oxygen-evolving complex to reduce the oxidative damage of chloroplasts and prevent the dissociation of thylakoid. The microfertilizer application also enhanced the electron receiving ability of the Q_B and plastoquinone (PQ) electronic pools, thereby increasing the ability of electron transfer from Q_A to Q_B . The number of reaction centers per unit area was promoted notably by the fertilization treatment.

Pseudostellaria heterophylla, in the Caryophyllaceae family, is a perennial herb that is well-known in China for its medicinal value. Its root tuber has medicinal and therapeutic functions such as invigorating the Qi and spleen, moisturizing the lungs, and relieving cough, and it has antihuman immunodeficiency virus infection, antidiabetic, and anticancer properties, among others (Sun et al., 2010; Yang et al., 2020a). With the increase in clinical demand and the

development of functional foods for *P. heterophylla*, its market demand has broadened steadily year by year, and its artificial cultivation areas have also expanded as well. As with other medicinal plant production, fertilizer application has become an important measure to improve the growth characteristics and yield of *P. heterophylla*. In recent years, the amount of N, P, K, and other elements of chemical fertilizer were high, and the use of organic fertilizer was very low in production.

The total biological output per unit area of arable land has continued to increase. However, the trace elements in soil were continuously consumed, thereby restricting the improvement of crop yield and nutrient quality (Verbruggen and Hermans, 2013).

Boron, Mo, and Cu are essential elements for plant growth and development (Wu et al., 2017a). These elements play important roles in regulating plant growth and development, especially in photosynthesis and other metabolic processes, and in organic matter transportation and distribution, and have been verified in many plants (Macedo et al., 2021). For example, one study showed that spraying B fertilizer on the leaves of *Raphanus sativus* effectively increased the chlorophyll content, enhanced photosynthesis, and promoted sugar metabolism during the flowering process (Wang et al., 2018). In *Beta vulgaris*, B fertilizer application outside the roots effectively improved its leaf performance and promoted root tuber expansion as well as its sugar content (Song et al., 2019). In wheat, Mo fertilizer application significantly increased the chlorophyll content, delayed the senescence, and prolonged the functional period of leaves, thereby improving the photosynthetic intensity, capacity, and time, and increasing the 1000 grain weight and yield of wheat (Li, 2019). In rice, Cu fertilizer application promoted the growth and increased yield of rice significantly (Mo, 2018). These results indicate that the application of B, Mo, and Cu alone could regulate the growth and development of plants significantly. Does their combination have a synergistic effect? To verify this scientific hypothesis, we examined B, Mo, and Cu combination fertilization to verify the synergistic effect between different microfertilizers and find the best fertilization scheme.

In the traditional cultivation and production of *P. heterophylla*, fertilizer application was often blind. People paid more attention to N–P–K fertilizers and ignored the use of microelement fertilizers. This led to a surplus of N–P–K and a depletion of microelements in the soil. Such practice reduced the fertilizer utilization rate, polluted the environment, and seriously affected the yield of *P. heterophylla* (Wu et al., 2017b). More N–P–K fertilizers were applied in the production practice of *P. heterophylla* (Zhang et al., 2015a), but there were few reports on the effects of the combined application of microfertilizers on the yield and photosynthetic characteristics of *P. heterophylla*. Therefore, we evaluated the effects of microelement fertilizers, particularly the combination of B, Mo, and Cu, on root yield of *P. heterophylla*. On the basis of optimizing the combination of B, Mo, and Cu for fertilizer application, the photosynthetic response mechanism of increased production was investigated further through the diurnal variation of photosynthesis and the chlorophyll fluorescence kinetics. Accordingly, results of this study provided a theoretical basis and application for the optimal ratio of B, Mo, and Cu as foliar fertilizers toward achieving high-efficiency cultivation in *P. heterophylla*.

Materials and Methods

Experimental field site. The experimental site was located at the Xiashu forest farm of Nanjing Forestry University (lat. 31°59'N, long. 119°E), Jiangsu Province, China, and the experiments were conducted between Nov. 2018 and June 2019. The soil type was yellow-brown soil with an organic matter content of 7.39 g·kg⁻¹, and total N and P contents of 0.704 and 0.146 g·kg⁻¹, respectively, and available P and K contents of 12.5 and 103.7 mg·kg⁻¹, respectively (Ma et al., 2017). Effective B, Mo, and Cu was 0.28, 0.1, and 0.99 mg·kg⁻¹, respectively.

Plant material and experimental design. The *P. heterophylla* seeds used in the study were provided by the forest station in Zherong County, Fujian Province. Strong, disease-free, and harmless seedlings of *P. heterophylla* were selected for cultivation. After soil preparation, ridges were constructed measuring 1600 cm in length, 100 cm in width, and 25 cm in height. Next, five furrows of ≈12 cm in depth with a row spacing of 15 cm were created. Drill sowing was performed at 100 g·m⁻², allowing a 4-cm spacing between plants.

The 3414 fertilizer application experiment design was adopted—namely, B fertilizer (Na₂B₄O₇·10H₂O), Mo fertilizer (H₈MoN₂O₄), and Cu fertilizer (CuSO₄·5H₂O): three factors and four levels with a total of 14 treatments. Three replicates were set for each treatment, and the experimental plot area was 2 m² (2 × 1 m). Tables 1 and 2 show the levels of microelement fertilizer application factors and the specific fertilizer application design, respectively. Treatment 1 (T1) served as control group (equal amount of distilled water). The trace element solution was sprayed on leaves twice every 10 d. The spraying time was in April and before 0900 HR on the day, and the spraying dose was based on leaf dripping. During the experiment, water and other environmental conditions remained consistent with standard field conditions.

Determination of growth indexes. seedling height and root length were measured with a

Table 1. The levels of microelement fertilization Boron (B), Molybdenum (Mo), and Copper (Cu) factors.

Factor levels	B (g·L ⁻¹)	Mo (g·L ⁻¹)	Cu (g·L ⁻¹)
0	0	0	0
1	0.50	0.04	0.05
2	1.00	0.08	0.10
3	1.50	0.12	0.15

Stanley 30-6-165 M tape (Stanley, Nanjing, China), whereas the ground and root diameters were measured with a 0- to 300-mm electronic vernier caliper (Deli, Nanjing, China). When the aboveground part of *P. heterophylla* was completely withered and yellow, all the samples were collected from each plot. The aboveground part was removed, and the underground part was washed and dried by air-drying. The fresh weight of root tubers was recorded and the yield was calculated by the mass of root tubers per unit area, with three replicates. Seedling height and ground diameter were measured with six replicates before harvest, whereas root length, root diameter, and yield per unit area were measured after harvest. Twenty root tubers were selected randomly to measure the root length and root diameter.

Determination of diurnal variation of photosynthesis. The diurnal variation of photosynthesis of *P. heterophylla* leaves was quantified by the standard leaf chamber of an Li-6400R portable photosynthesis system (Li-COR Bio-sciences, Lincoln, NE) on a sunny day in mid-May. Measurements were conducted in a 6-cm² leaf chamber under the condition of an open gas system, uncontrolled ambient CO₂ concentration and air temperature, and natural light. Six leaves that were deemed healthy, functional, and of a similar size were identified in each treatment and measured. Measurements were taken at 2-h intervals from 0700 to 1700 HR at the same position. The diurnal changes in net photosynthetic rate (P_n), stomatal conductance (g_s), intercellular CO₂ concentration (C_i), and transpiration rate (T_r) were measured simultaneously using the same method. The data were recorded when the rate of CO₂ uptake had stabilized.

Determination of fast chlorophyll fluorescence induction kinetic curve. The parameters of chlorophyll fluorescence induction kinetics were measured using a continuous excitation fluorometer (Handy-PEA; Hansatech, UK) in mid-May. The leaves were first adapted in the dark for 30 min, then exposed to saturated, pulsed light. The fast fluorescence kinetic induction curves (OJIP) were measured with the Handy-PEA and analyzed using the analysis of fast fluorescence kinetic induction curve (JIP-test) (Strasser, 1997). Table 3 shows the parameters of chlorophyll fluorescence induction kinetics.

Statistical analysis. Data processing and statistics were done in Excel 2010 and SPSS 18.0 statistical software (Version 18.0; IBM Corp., Armonk, NY). Post hoc comparisons of means were evaluated using Duncan's new complex range method and were measured at the 5% significance level.

Results

Optimization of fertilizer amounts of B, Mo, and Cu in *P. heterophylla*

Table 4 shows the effects of the combined application of B, Mo, and Cu on seedling height, ground diameter, root length, root diameter, and yield per unit area of *P. heterophylla* in varying degrees. Of the 14 treatments, T9 (B₂Mo₂Cu₁) showed the best positive effect for growth indicators except seedling height. Compared with T1 (control, B₀Mo₀Cu₀), T9 values for the growth indicators were increased by 16.48%, 10.10%, 13.63%, 28.19%, and 35.17%, respectively. The results showed that the optimal combined application of B, Mo, and Cu influenced the growth of the aboveground and underground parts of *P. heterophylla* significantly and positively. The optimal B, Mo, and Cu combination not only increased the yield per unit area effectively of *P. heterophylla* root, but also promoted significantly the quality of the single root tuber (root length and diameter).

Table 2. Boron (B), Molybdenum (Mo), and Copper (Cu) application scheme.

Treatment no.	Treatment combination	Fertilizer level			Fertilizer dose (g·L ⁻¹)		
		B	Mo	Cu	B	Mo	Cu
1	B ₀ Mo ₀ Cu ₀	0	0	0	0	0	0
2	B ₀ Mo ₂ Cu ₂	0	2	2	0	0.08	0.10
3	B ₁ Mo ₂ Cu ₂	1	2	2	0.50	0.08	0.10
4	B ₂ Mo ₀ Cu ₂	2	0	2	1	0	0.10
5	B ₂ Mo ₁ Cu ₂	2	1	2	1	0.04	0.10
6	B ₂ Mo ₂ Cu ₂	2	2	2	1	0.08	0.10
7	B ₂ Mo ₃ Cu ₂	2	3	2	1	0.12	0.10
8	B ₂ Mo ₂ Cu ₀	2	2	0	1	0.08	0
9	B ₂ Mo ₂ Cu ₁	2	2	1	1	0.08	0.05
10	B ₂ Mo ₂ Cu ₃	2	2	3	1	0.08	0.15
11	B ₃ Mo ₂ Cu ₂	3	2	2	1.50	0.08	0.10
12	B ₁ Mo ₁ Cu ₂	1	1	2	0.50	0.04	0.10
13	B ₁ Mo ₂ Cu ₁	1	2	1	0.50	0.08	0.05
14	B ₂ Mo ₁ Cu ₁	2	1	1	1	0.04	0.05

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Table 3. The parameters of chlorophyll fluorescence induction kinetics.

F_v/F_m	maximum photochemical efficiency of PSII
PI_{abs}	photochemical performance index
ϕPo	maximum quantum efficiency of PSII
Ψ_o	electron transfer efficiency
ϕEo	quantum yield of electron transfer
ϕDo	quantum ratio of heat dissipation
ABS/RC	energies absorbed by the unit reaction center
TRo/RC	energies captured by the unit reaction center
ETo/RC	energies used for electron transfer by the unit reaction center
TRo/RC	energies used for heat dissipation by the unit reaction center
RC/CSO	density of reaction center
V_L	L point relative variable fluorescence; $V_L = (F_t - F_o) / (F_K - F_o)$, where L is 0.15 ms
V_K	K point relative variable fluorescence; $V_K = (F_t - F_o) / (F_J - F_o)$, where K is 0.3 ms
V_J	J point relative variable fluorescence; $V_J = (F_t - F_o) / (F_m - F_o)$, where J is 2 ms
V_I	I point relative variable fluorescence; $V_I = (F_t - F_o) / (F_m - F_o)$, where I is 30 ms

PSII = photosystem II.

Effects of the combined application of B, Mo, and Cu on the diurnal variation of photosynthesis in *P. heterophylla*

To explore further the physiological mechanism of *P. heterophylla* treated with the B, Mo, and Cu combinational application from a photosynthetic perspective, the best treatment for increasing production (T9) and the control group (T1) were selected as materials to analyze further and compare the characteristics of the diurnal variation of photosynthesis and photosystem II (PSII) rapid fluorescence induction kinetics.

Environmental factors. The photosynthetic photon flux density was only $500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at 0700 HR. After 0700 HR, it rose significantly and peaked at $1500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ around 1300 HR, followed by a marked decrease thereafter, dropping to less than $600 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at 1700 HR. The air temperature presented a single-

peak curve, reaching its maximum value of 31.76°C at 1300 HR, and the average temperature was 27.03°C . Diurnal fluctuations in air relative humidity decreased over time, with a maximum value of 56% at 0700 HR followed by a rapid decrease, dropping to 38% by 1300 HR. The trend in ambient CO_2 concentration was different from the other environmental factors. It vacillated between 407 and $413 \mu\text{mol}\cdot\text{mol}^{-1}$ between sunrise and sunset.

Diurnal photosynthetic variation. Figure 1A shows that the *P. heterophylla* leaves in T1 had a bimodal response in P_n , displaying a discernable photosynthetic midday depression (1300 HR), with two peaks occurring at 0900 and 1500 HR, with the first peak being higher than the second. Compared with T1, T9 showed no change in bimodal pattern. However, the whole-day P_n increased significantly, including the daily mean value (65.55%), peak values (51.80%, 107.76%), and valley value (104.63%) ($P < 0.05$), thereby effectively circumventing the photosynthetic midday depression experience.

Figure 1B–D showed that the daily variation trend in g_s in *P. heterophylla* was basically consistent with that of P_n . Both T1 and T9 showed double-peak changes. However, T9 increased significantly the daily mean value, peak value, and valley value of g_s . The diurnal variation of C_i was contrary to that of P_n and g_s . In general, the change trend of C_i was the W type, and it showed a peak value during the photosynthetic midday depression. T9 reduced C_i significantly in the process of daily change, including daily mean, valley value, and peak value. The daily T_r profile showed a rapid increase to the daily maximum at midday (1300 HR) and then a gradual reduction in as the day proceeded to dusk. Moreover, T9 showed a pronounced positive influence on the whole-day T_r compared with T1.

Effect of the combined application of B, Mo, and Cu on the characteristics of rapid fluorescence induction kinetics

PSII photochemical activity. Figure 2 shows that, compared with the control group (T1), the T9 fertilizer application increased the maximum photochemical efficiency (F_v/F_m)

and the photochemical performance index (PI_{abs}) of PSII. At $P < 0.05$, F_v/F_m and PI_{abs} was increased by 5.23% and 163.25%, respectively. The increase in PI_{abs} was significantly greater than that of F_v/F_m .

Figure 3 shows the effect of fertilizer application on the energy distribution ratio of the PSII reaction center in *P. heterophylla*. At $P < 0.05$, the maximum quantum efficiency (ϕPo , 5.26%), electron transfer efficiency (Ψ_o , 20.25%) and quantum yield of electron transfer (ϕEo , 27.20%) of PSII based on T9 were significantly greater than those of the control group (T1). The quantum ratio of heat dissipation (ϕDo) was significantly less than T1, which was decreased by 13.82% ($P < 0.05$).

Figure 4 shows the increases in the energy of absorption (ABS/RC, 15.29%), capture (TRo/RC, 8.14%), and electron transfer (ETo/RC, 12.33%) per unit reaction center in T9 compared with T1. The energy used for heat dissipation by unit reaction center (Dio/RC) was decreased by 28.05% compared with that of T1. At the same time, T9 showed an increase in the density of the PSII reaction center (RC/CSO, 68.62%) of *P. heterophylla* leaves compared with T1 ($P < 0.05$).

The relative variable fluorescence of the L point and K point on the OJIP curve. Significant differences were observed in the relative variable fluorescence of the L point (V_L ; 0.15 ms on the standardized O–K curve) and K point (V_K ; 0.3 ms on the standardized O–J curve) in *P. heterophylla* leaves between T1 (control) and T9 (with microelement fertilizers). Figure 5 illustrates the difference values of the standardized O–K and O–J curves between T1 and T9. It should be noted that the relative variable fluorescence of the L and K points was quite different in the two groups. Quantitative analysis at $P < 0.05$ showed that the V_L and V_K of T9 was decreased by 15.81% and 26.33%, respectively, compared with T1.

The relative variable fluorescence of the J point and I point on the OJIP curve. After the OJIP curve was standardized by O–P (Fig. 6), T9 exhibited a reduction in relative variable fluorescence of the J point (V_J ; 2 ms on the standardized O–P curve) and I point (V_I ; 30 ms on the standardized O–P curve) of *P. heterophylla* leaves. The difference values of the standardized O–P curves of T1 and T9 showed that the reduction in V_J was greater than that of V_I . The results of quantitative analysis showed that V_J and V_I of T9 decreased by 14.23% and 4.58% compared with T1, respectively. The change in V_J was significant, but the change in V_I was not obvious.

Discussion

Optimization of B, Mo, and Cu combined fertilizer in *P. heterophylla*. The 3414 fertilizer application experiment design, a kind of quadratic regression D-optimal design, has the advantages of low frequency treatment and high efficiency. It also meets the professional requirements of fertilizer experiments and decision making in fertilizer application and use (Ma et al., 2019; Yang et al., 2020b).

Table 4. Effects of combined applications of Boron (B), Molybdenum (Mo), and Copper (Cu) on the growth indexes of *P. heterophylla*.

No.	Seedling ht (cm)	Ground diam (mm)	Root length (cm)	Root diam (mm)	Yield ($\text{g}\cdot\text{m}^{-2}$)
1	12.62 ± 0.73 d	1.98 ± 0.07 b	8.29 ± 0.01 d	5.64 ± 0.28 ef	335.16 ± 5.00 j
2	12.75 ± 0.53 d	2.00 ± 0.11 b	8.47 ± 0.10 cd	6.04 ± 0.49 cde	369.80 ± 28.80 h
3	13.50 ± 0.90 bcd	2.00 ± 0.03 b	8.85 ± 0.19 abcd	6.18 ± 0.26 cd	399.95 ± 8.10 g
4	13.85 ± 0.26 abc	2.00 ± 0.08 b	8.77 ± 0.97 bcd	5.52 ± 0.23 f	401.47 ± 9.63 fg
5	14.02 ± 0.70 ab	2.02 ± 0.10 b	8.97 ± 0.23 abc	6.98 ± 0.11 ab	429.25 ± 1.27 cd
6	13.98 ± 0.96 ab	2.01 ± 0.06 b	9.11 ± 0.08 ab	6.25 ± 0.24 c	421.47 ± 12.64 cd
7	13.00 ± 0.74 cd	1.94 ± 0.06 b	8.27 ± 0.06 d	5.78 ± 0.36 def	391.48 ± 1.05 g
8	13.98 ± 1.00 ab	1.99 ± 0.11 b	9.32 ± 0.27 ab	7.01 ± 0.28 ab	420.04 ± 11.85 de
9	14.70 ± 1.01 a	2.18 ± 0.14 a	9.42 ± 0.19 a	7.23 ± 0.07 a	453.02 ± 22.02 a
10	12.67 ± 0.92 d	1.98 ± 0.06 b	8.32 ± 0.10 d	6.18 ± 0.17 cd	358.69 ± 12.99 i
11	14.72 ± 0.81 a	2.15 ± 0.08 a	9.35 ± 0.36 ab	6.72 ± 0.22 b	440.44 ± 20.02 b
12	13.88 ± 0.84 abc	2.02 ± 0.09 b	8.97 ± 0.08 abc	6.82 ± 0.02 ab	410.68 ± 19.36 ef
13	13.37 ± 1.10 bcd	1.98 ± 0.12 b	8.40 ± 0.41 cd	6.96 ± 0.27 ab	395.45 ± 8.75 g
14	14.05 ± 0.46 ab	2.15 ± 0.12 a	9.23 ± 0.67 ab	7.17 ± 0.24 a	430.47 ± 28.56 bc

Different lowercase letters within a column indicate a significant difference at the 0.05 level between different treatments. Seedling height and ground diameter ($n = 6$); root length and root diameter ($n = 20$); yield ($n = 3$).

Fig. 1. Effects of the combined application of Boron (B), Molybdenum (Mo), and Copper (Cu) on (A) diurnal variation of net photosynthetic rate (P_n), (B) stomatal conductance (g_s), (C) intercellular CO_2 concentration (C_i), and (D) transpiration rate (T_r) in *Pseudostellaria heterophylla* ($n = 6$). Different lowercase letters indicate significant differences between treatments ($P < 0.05$). T1 = B, 0 $\text{g}\cdot\text{L}^{-1}$; Mo, 0 $\text{g}\cdot\text{L}^{-1}$; Cu, 0 $\text{g}\cdot\text{L}^{-1}$. T9 = B, 1 $\text{g}\cdot\text{L}^{-1}$; Mo, 0.08 $\text{g}\cdot\text{L}^{-1}$; Cu, 0.05 $\text{g}\cdot\text{L}^{-1}$.

In recent years, the 3414 design has been gradually adapted into the practical production of medicinal plants. For example, the combined application of B–Zn–Mo 3414 effectively improved the root quality of *Bupleurum chinense* (Meng et al., 2014), and N–P–K 3414 use showed a significant impact on the yield of *Agastache rugosa* (Xue et al., 2018). In addition, the 3414 fertilizer design has been tested on various types of Chinese medicinal plants, such as *Panax notoginseng* (Xia et al., 2016), *Rheum tanguticum* (Qi et al., 2015), and *Atractylodes lancea* (Hu et al., 2017), and showed significant effects. Therefore, the reasonable fertilizer application can provide a theoretical basis for standardized cultivation to achieve the goals of high yield and high-quality plants.

The combined application of microfertilizer is an important measure to promote plant growth and yield (Wang et al., 2020). It has the advantages of less dosage, low cost, no pollution, quick effect, and convenient operation. Different plants have different requirements and tolerances to trace elements; a too-low or too-high application dosage would affect fertilizer efficiency and even damage the soil environment. Boron, Mo, and Cu are trace mineral elements necessary for plant growth and development. They participate in the regulation of photosynthesis directly or indirectly, and appropriate application could effectively increase crop yield (Faisal and Muhammad, 2019). A few studies of their application to medicinal plants have been conducted, but none have been reported on

application to *P. heterophylla*. The results of our study show that the combined application of B, Mo, and Cu via the 3414 experimental design could significantly improve the root tuber yield of *P. heterophylla*. Our results provide an important basis for the combined B, Mo, and Cu fertilizer ratio toward high-yield cultivation of *P. heterophylla*, and further proved the effectiveness of the 3414 experimental design.

The optimal combination of microfertilizers mainly improved the P_n capacity of *P. heterophylla* by improving nonstomatal limitation. Analysis of diurnal variation of photosynthesis is one of the important means of exploring the effects of environmental factors on plant growth and metabolism (Lamprey et al., 2017). The characteristic parameters of

Fig. 2. Effects of the combined application of Boron (B), Molybdenum (Mo), and Copper (Cu) on the maximum photochemical efficiency of PSII (F_v/F_m) and the photochemical performance index (Plabs) in *P. heterophylla*. Different lowercase letters indicate significant differences between treatments ($P < 0.05$; $n = 6$). T1 = B, 0 $\text{g}\cdot\text{L}^{-1}$; Mo, 0 $\text{g}\cdot\text{L}^{-1}$; Cu, 0 $\text{g}\cdot\text{L}^{-1}$. T9 = B, 1 $\text{g}\cdot\text{L}^{-1}$; Mo, 0.08 $\text{g}\cdot\text{L}^{-1}$; Cu, 0.05 $\text{g}\cdot\text{L}^{-1}$.

Fig. 3. Effects of the combined application of Boron (B), Molybdenum (Mo), and Copper (Cu) on the energy distribution ratio of the photosystem II (PSII) reaction center in *P. heterophylla*. Different lowercase letters indicate significant differences between treatments ($P < 0.05$; $n = 6$). T1 = B, 0 g·L⁻¹; Mo, 0 g·L⁻¹; Cu, 0 g·L⁻¹. T9 = B, 1 g·L⁻¹; Mo, 0.08 g·L⁻¹; Cu, 0.05 g·L⁻¹.

diurnal variation of photosynthesis reflect the continuous ability of material accumulation and physiological metabolism of plants in a day. More importantly, they help when determining and analyzing the limiting factors in photosynthesis (Liu et al., 2019). Our study showed that the diurnal variation of P_n in *P. heterophylla* leaves had an obvious photosynthetic midday depression in the current environmental conditions, when the local temperature had not yet reached a high in mid-May. This indicates that *P. heterophylla* has poor tolerance to high temperatures and strong light. This result is in accordance with the characteristics of *P. heterophylla*'s preference for a cool environment under natural growth conditions. During the photosynthetic midday depression period, P_n and g_s of T1 (control) decreased significantly, whereas C_i increased significantly, indicating that the decrease in P_n capacity was caused mainly by nonstomatal factors (Farquhar and Sharkey, 1982). When compared with T1, T9 (the optimized treatment) did not change the bimodal variation trend of *P. heterophylla* P_n , but increased P_n and g_s , and decreased C_i during the photosynthetic midday depression period. Our results indicate that the applied combined

fertilizer alleviated the photosynthetic midday depression of *P. heterophylla* leaves by improving nonstomatal factors. At the same time, judging from the diurnal variation of photosynthesis throughout the whole day, T9 showed a significant increase in the daily mean of P_n and g_s , whereas C_i was decreased significantly. Our results also show that the optimal combination of micronutrient fertilizer promoted the P_n capacity of *P. heterophylla* by improving nonstomatal limitation throughout the day.

The optimal combination of microfertilizers improved PSII photochemical activity and alleviated photoinhibition. PSII is the primary and main part of photoinhibition when plants are exposed to high temperatures, strong light, and other environmental stresses (Yu et al., 2020). In the light reaction system, PSII is affected more easily by the internal and external environment than photosystem I. Because the JIP test can analyze the functional state and specific information of each part in the primary photochemical reaction process of PSII quickly and nondestructively, it was suitable for exploring further the mechanism of improving the nonstomatal limitation of *P. heterophylla* by combined application of microfertilizers.

Both F_v/F_m and PI_{abs} are important indicators of plant photoinhibition (Kalaji et al., 2016). F_v/F_m was about 0.8 when plants grew normally. In our study, the F_v/F_m value of *P. heterophylla* in T1 was significantly less than 0.75, indicating that PSII had photoinhibition. This observation further proved that *P. heterophylla* was not resistant to strong light and high temperatures. Compared with T1, plant leaves in T9 showed a significant increase in F_v/F_m and PI_{abs} . T9 also demonstrated a significant increase in the maximum P_n rate (peak value) and the valley value at the mid-day depression (Fig. 1). These results indicate that the combined fertilizers applied alleviated the photoinhibition of *P. heterophylla* leaves and improved its photosynthetic performance.

The results of energy distribution ratio parameters (ϕPo , ψo , ϕEo , and ϕDo) in T9 showed an increase in the absorption and capture of light energy and the energy ratio for electron transfer of PSII, but a decrease in the heat dissipation ratio. Thus, plants in T9 enhanced the photochemical activity of PSII and alleviated photoinhibition by enhancing the photochemical utilization of light energy, rather than by heat dissipation. The results of specific activity parameters (ABS/RC, TRo/RC, ETo/RC, and DIo/RC) and density parameters (RC/CSO) of the PSII reaction center further shows that the microfertilizers applied enhanced the activity of the PSII unit reaction center, and increased the number of reaction centers per unit area of leaves, thus improving the photochemical activity of PSII in whole leaves.

The photochemical activity of the PSII reaction center was reflected mainly in the electron transfer efficiency of PSII. This is related primarily to the electron supply capacity of the PSII electron donor side and the electron transfer capacity of the PSII electron acceptor side (Jedrowski and Brueggemann, 2015; Khoshbakht et al., 2018). The change in relative variable fluorescence at the inflection point in the OJIP curve is helpful to analyze further the electron transport of PSII and to explore the mechanism of enhancing the photochemical activity of *P. heterophylla* by foliar microfertilizer application. The increase in K point relative variable fluorescence on the OJIP curve is a specific marker of the

Fig. 4. Effects of the combined application of Boron (B), Molybdenum (Mo), and Copper (Cu) on the specific activity parameters and density of the photosystem II (PSII) reaction center in *P. heterophylla*. Different lowercase letters indicate significant differences between treatments ($P < 0.05$; $n = 6$). T1 = B, 0 g·L⁻¹; Mo, 0 g·L⁻¹; Cu, 0 g·L⁻¹. T9 = B, 1 g·L⁻¹; Mo, 0.08 g·L⁻¹; Cu, 0.05 g·L⁻¹.

Fig. 5. Effects of the combined application of Boron (B), Molybdenum (Mo), and Copper (Cu) on the standardized O-K and O-J curves in *P. heterophylla* leaves. Different lowercase letters indicate significant differences between treatments ($P < 0.05$; $n = 6$). T1 = B, 0 g·L⁻¹; Mo, 0 g·L⁻¹; Cu, 0 g·L⁻¹. T9 = B, 1 g·L⁻¹; Mo, 0.08 g·L⁻¹; Cu, 0.05 g·L⁻¹.

damage to the oxygen-evolving complex (OEC). As OEC activity is inhibited, the water-splitting function becomes disordered and a large amount of H₂O₂ is produced in the chloroplasts. H₂O₂ aggravates the oxidative damage of chloroplast membrane lipids and increases membrane permeability. With the increase of membrane lipid peroxidation in the chloroplasts, membrane lipids and membrane proteins are free to form an osmophilic body—namely, liposome. The formation of liposomes results from degradation and aggregation of chloroplast thylakoids, and the rise of the L point relative variable fluorescence (0.15 ms) on the OJIP curve is a specific marker of thylakoid dissociation. Absorption, transmission, and transformation of light energy by plant leaves are carried out on the thylakoid membrane of chloroplast, and the PSII protein complex is embedded directly in the thylakoid membrane. Therefore, the destruction of PSII function is related directly to the dissociation of thylakoid. The V_L and V_K of *P. heterophylla* leaves were decreased significantly in T9, suggesting

that microfertilizer application could protect OEC activity to reduce oxidative damage to the chloroplasts, prevent dissociation of thylakoid, and increase the tight connection between protein subunits in the PSII complex to maintain the normal physiological function of PSII and reduce the degree of photoinhibition (Hao et al., 2019). The relative variable fluorescence of the J point on the OJIP curve represents the number of closed reaction centers or the reduced amount of Q_A. Q_A is often reduced excessively and Q_A⁻ accumulates greatly under adverse conditions—namely, V_J tends to increase (Zhang et al., 2015b). The accumulation of Q_A⁻ is a result of the inhibition of the electron transfer from Q_A⁻ to Q_B after pheophytin transfers electrons to Q_A to produce Q_A⁻ (Zhang et al., 2015b). Therefore, the increase in V_J is also a specific indicator that the electron transfer from Q_A to Q_B on the PSII receptor side is blocked. In our study, the foliar microfertilizer treatment (T9) reduced the V_J of *P. heterophylla* leaves, suggesting that elements from the microfertilizers could relatively increase the electron transfer

capacity of Q_A to Q_B on the PSII receptor side. The change in the relative variable fluorescence of I point at 30 ms reflects the heterogeneity of the PQ pool during the electron transfer from Q_A to Q_B (Strasser, 1997). The microfertilizer application reduced the V_I of *P. heterophylla* leaves, suggesting that microfertilizers could increase the ability of the PQ pool to accept electrons, which is also one of the reasons for promoting the electron transfer from Q_A⁻ to Q_B. The microfertilizer treatment both increased the electron supply capacity of the PSII donor side and the electron transfer capacity of the PSII receptor side, and especially protected the activity of OEC effectively on the donor side.

Conclusion

Our results show that the 3414 experimental design helped optimized the combined microfertilizer ratio of B, Mo, and Cu for application on the leaf surface of *P. heterophylla*. We also demonstrated that microfertilizer application can promote the growth of *P.*

Fig. 6. Effects of the combined application of Boron (B), Molybdenum (Mo), and Copper (Cu) on the standardized O-P curve in *P. heterophylla* leaves. Different lowercase letters indicate significant differences between treatments ($P < 0.05$; $n = 6$). T1 = B, 0 g·L⁻¹; Mo, 0 g·L⁻¹; Cu, 0 g·L⁻¹. T9 = B, 1 g·L⁻¹; Mo, 0.08 g·L⁻¹; Cu, 0.05 g·L⁻¹.

heterophylla significantly and increase its root tuber yield per unit area. Moreover, the use and application of the optimal ratio of micro-fertilizers enhanced the P_n capacity significantly during the process of diurnal variation of photosynthesis by improving nonstomatal limitation.

Optimal application of combined B, Mo, and Cu fertilizers promoted the absorption, capture, and conversion of light energy and efficiency of electron transfer by *P. heterophylla* PSII, and alleviated the photoinhibition caused by excess excitation energy. The stability in structure and function of OEC on donor side of PSII, the enhancement of electron transfer ability of Q_A to Q_B on receptor side, and the increased number of reaction centers per unit area might be the main mechanisms for enhancing photochemical activity of PSII and improving photosynthetic performance of *P. heterophylla* leaves. The results of this study provided an important basis for the ratio of B, Mo, and Cu fertilizers to use and apply toward high-yield cultivation and its physiological mechanism in *P. heterophylla*.

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