Effects of Nitrogen, Phosphorus, and Potassium Addition on Soil Microbial Functional Diversity in Podocarpus macrophyllus: Postprint

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Abstract

By investigating the effects of different nitrogen, phosphorus, and potassium nutrient level additions on soil microbial biomass and diversity and their carbon source utilization characteristics in Podocarpus macrophyllus, this study reveals the response patterns of soil microorganisms to different NPK nutrient levels, providing a theoretical basis for fertilization and management of P. macrophyllus from a microbial perspective. Using two-year-old P. macrophyllus seedlings as the experimental tree species, an L9 orthogonal experiment was employed to control the NPK nutrient level gradients in potted soil. The dilution plate spreading method and Biolog-ECO microplate method were used to explore the effects of different soil nutrient levels on soil microbial biomass and community diversity and their utilization characteristics of six carbon sources. The results showed that: (1) With increasing nitrogen addition, soil bacterial ($P < 0.05$) and actinomycete numbers ($P < 0.001$) decreased, while fungal ($P < 0.001$) and nitrogen-fixing bacterial numbers ($P < 0.01$) significantly increased. The Pielou index ($P < 0.001$) of the soil microbial community decreased, while the Simpson index ($P < 0.05$) and McIntosh index ($P < 0.001$) increased, thereby reducing the utilization intensity of soil microorganisms for the six carbon sources, particularly significantly decreasing the utilization intensity for difficult-to-utilize carbon sources including amines ($P < 0.001$), carboxylic acids ($P < 0.001$), polymers ($P < 0.001$), and other compounds ($P < 0.001$). (2) Increasing phosphorus addition significantly reduced the Shannon index ($P < 0.05$) of the soil microbial community. (3) Increasing potassium addition significantly reduced the Shannon index ($P < 0.05$) and Pielou index ($P < 0.05$) of the soil microbial community, as well as the utilization intensity of the microbial community for two readily utilizable carbon sources: carbohydrates ($P < 0.001$) and amino acids ($P < 0.01$). In summary, nitrogen and potassium additions are the main factors affecting the functional diversity of soil microbial communities in P. macrophyllus. During cultivation of P. macrophyllus, attention should be paid to applying fertilizer in small amounts and multiple times, reducing nitrogen and potassium addition levels, and appropriately increasing phosphorus addition to promote the growth and sustainable cultivation of P. macrophyllus.

Full Text

Preamble

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Effects of nitrogen, phosphorus and potassium additions on functional diversity of soil microorganisms in *Podocarpus macrophyllus*

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Abstract

This study investigated the effects of different nitrogen (N), phosphorus (P), and potassium (K) nutrient addition levels on soil microbial biomass, diversity, and carbon source utilization in *Podocarpus macrophyllus*, aiming to reveal the response patterns of soil microorganisms to varying NPK nutrient levels and provide a theoretical basis for fertilization and management of *P. macrophyllus* from a microbial perspective. Using two-year-old *P. macrophyllus* seedlings, we controlled NPK nutrient gradients in potted soil using an L9 orthogonal experimental design and employed dilution plate coating and Biolog-ECO microplate methods to explore how different soil nutrient levels affected soil microbial biomass, community diversity, and utilization characteristics of six carbon sources. The results showed that: (1) With increasing N addition, soil bacterial ($P < 0.05$) and actinomycete ($P < 0.001$) numbers decreased, while fungal ($P < 0.001$) and nitrogen-fixing bacterial ($P < 0.01$) numbers increased significantly. The Pielou index ($P < 0.001$) of the soil microbial community decreased, while the Simpson index ($P < 0.05$) and McIntosh index ($P < 0.001$) increased, thereby reducing the intensity of utilization of six carbon sources by soil microorganisms, particularly the utilization intensity of difficult-to-use carbon sources including amines ($P < 0.001$), carboxylic acids ($P < 0.001$), polymers ($P < 0.001$), and other compounds ($P < 0.001$). (2) Increased P addition significantly reduced the Shannon index of the soil microbial community ($P < 0.05$). (3) Increased K addition significantly reduced the Shannon index ($P < 0.05$) and Pielou index ($P < 0.05$) of the soil microbial community, as well as the utilization intensity of two easily available carbon sources—carbohydrates ($P < 0.001$) and amino acids ($P < 0.01$). In conclusion, N addition and K addition are the main factors affecting the functional diversity of soil microbial communities in *P. macrophyllus*. Cultivation of *P. macrophyllus* should employ small, frequent fertilization applications, reduce N and K addition rates, and appropriately increase P addition to promote growth and sustainable cultivation.

Keywords: L9 orthogonal test, Biolog-ECO, *Podocarpus macrophyllus*, fertilization, carbon source utilization, microbial community functional diversity

Introduction

Podocarpus macrophyllus is a perennial evergreen coniferous tree belonging to the family Podocarpaceae and genus *Podocarpus*, mainly distributed in various provinces south of the Yangtze River in China (Mill, 2003). It is widely used in landscaping, bonsai appreciation, and the pharmaceutical industry, possessing high economic value (Huo et al., 2023). In recent years, with improving living standards, *P. macrophyllus* bonsai has become increasingly popular. *P. macrophyllus* grows slowly and often requires a long time to develop into a bonsai specimen (Aiba & Kitayama, 1999). Scientific fertilization can not only promote rapid seedling growth and shorten the time required for bonsai development but also significantly improve seedling quality and sustainable cultivation.

Fertilization promotes seedling growth and enhances soil fertility while playing a crucial regulatory role in soil microbial community abundance and structure (Li, 2021). Soil microorganisms, as the most sensitive and important components of soil ecosystems (Bardgett et al., 2005), largely determine the rate of material cycling and reflect soil conditions to a certain extent (Kramer et al., 2013), including organic matter decomposition and simple substance resynthesis capacity (Cusack et al., 2011), nutrient cycling, and biological nitrogen fixation (Hemkemeyer et al., 2021). The intensity of carbon source utilization by soil microorganisms is an important indicator for measuring soil quality and health and evaluating soil ecosystem stability (Yang et al., 2013). Studies have shown that additions of N, P, and K nutrients can significantly increase plant soil microbial biomass (Jangid et al., 2008; Li et al., 2015; Zhang et al., 2018), microbial diversity (Kracmarova et al., 2020), average well color development, and carbon source utilization intensity (Jiang et al., 2019).

Currently, few studies have examined the effects of nutrient addition on soil microbial community functional diversity in *P. macrophyllus*. To clarify the effects of different NPK addition rates on *P. macrophyllus* seedling cultivation and determine the optimal NPK application rates, this study was conducted at the teaching and research base of the College of Forestry, Guangxi University. Using an L9 orthogonal experimental design (Jiang et al., 2021), dilution plate coating method, and Biolog-ECO microplate method (Huang, 2019; Li et al., 2022; Ochieno, 2022; Kodadinne et al., 2022), we investigated soil microbial community carbon source utilization activity under different NPK addition treatments. Combined with L9 range analysis, redundancy analysis (RDA), and canonical correlation analysis (CCA), we analyzed soil microbial community functional diversity to provide a theoretical basis for fertilization and management of *P. macrophyllus* from a microbial perspective.

1.1 Experimental Site Overview

The experimental site was located at the teaching and research base of the College of Forestry, Guangxi University in Nanning, China (108°12 E, 22°50 N). The region has a south subtropical monsoon climate with abundant sunshine throughout the year, an average annual temperature of approximately 21.8 °C, annual rainfall of about 1,350 mm, and average relative humidity of 76%.

1.2 Experimental Design

This experiment employed a pot culture method using two-year-old *P. macrophyllus* seedlings as test materials, with an average height of 50 cm and average ground diameter of 0.5 cm. The pots measured 45 cm in height and 20 cm in inner diameter. The test soil was a 1:1 mixture of forest raw soil and river sand. The forest raw soil was relatively infertile, which better illustrated the effects of nutrient addition on soil microbial functional diversity. Detailed basic physicochemical properties of the soil are shown in Table 1 . In March 2018, each pot was filled with 8 kg of soil and planted with one seedling. An L9 orthogonal experimental design was adopted (Jiang et al., 2021), with three gradient levels each for N, P, and K addition, and 15 replicates per treatment. Details of each component and addition amount are shown in Table 2 . Urea ($N \geq 46\%$) was used for N addition, calcium superphosphate ($P_2O_5 \geq 12\%$) for P addition, and potassium chloride ($K_2O \geq 60\%$) for K addition, all of which are quick-acting fertilizers. Fertilizers were mixed with soil according to the settings in Table 2 and placed in pots. The potted plants were watered three times per week to ensure normal seedling growth. In July 2018, plant height and ground diameter were measured, and soil from the 0–20 cm layer was collected. Soil from every five pots was combined into one sample to eliminate individual differences, with three mixed samples per treatment for measurement.

1.3 Determination of Soil Microbial Biomass Carbon and Nitrogen

Soil microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were determined using the chloroform fumigation method (Könönen et al., 2018). Ten grams of fresh soil sample were placed in a 27 °C incubator at 65% humidity for 24 h, then transferred to a well-sealed vacuum desiccator containing a small beaker with 10 mL of chloroform and another with 50 mL of dilute NaOH solution. A vacuum pump was used to boil the chloroform vigorously for 5 min, after which the system was closed and incubated in the dark at 25 °C for 24 h. Unfumigated samples served as controls. After incubation, 50 mL of 0.5 $\mathrm{mol \cdot L^{-1}}$ K_2SO_4 solution was added to each sample, followed by thorough shaking and filtration. The clarified solution was immediately analyzed for microbial biomass carbon and nitrogen using a TOC analyzer.

Where: BC is microbial biomass carbon; EC is microbial biomass carbon in fumigated samples; E_0 is microbial biomass carbon in unfumigated samples; KEC is the conversion coefficient 0.40; BN is microbial biomass nitrogen; EN is microbial biomass nitrogen in fumigated samples; E_1 is microbial biomass nitrogen in unfumigated samples; KEN is the conversion coefficient 0.45.

1.4 Determination of Soil Microbial Quantities

Soil microbial quantities were determined using the dilution plate coating method, primarily measuring bacteria, fungi, actinomycetes (ACT), and nitrogen-fixing bacteria (NFB). Bacteria were cultured on beef extract peptone medium, actinomycetes on Gause' s No. 1 medium, fungi on Martin' s medium, and nitrogen-fixing bacteria on mannitol agar medium. Bacteria were cultured for 1-2 days, fungi for 3-5 days, nitrogen-fixing bacteria for 4-5 days, and actinomycetes for 5-7 days, after which plates were removed for colony counting.

1.5 Determination of Soil Microbial Functional Diversity

Soil microbial community functional diversity was measured using the Biolog-ECO microplate method. Ten grams of processed soil sample were added to sterilized physiological saline, shaken thoroughly, and the supernatant was diluted to obtain a 10^{-3} bacterial suspension, which was then inoculated into Biolog EcoPlates and incubated at 25 °C in darkness. Absorbance values at 590 nm and 720 nm were measured every 24 h using an automated microbial identification system. After measurement, the average well color development (AWCD) was calculated for each time point. After stable cultivation, the Shannon index (H), Simpson index (D), McIntosh index (U), and Pielou index (E) were calculated to evaluate the dominance of common species, species richness, and evenness of the soil microbial community (Fang et al., 2019).

Where: C is the absorbance value of 31 carbon source wells; R is the absorbance value of control wells; P is the absorbance value of each well divided by the sum of all well absorbance values; N is the relative absorbance value of the i-th well; and S is the number of carbon source types utilized by microorganisms.

1.6 Determination of Plant Height and Ground Diameter

Plant height was measured using a steel ruler, and ground diameter was measured using vernier calipers.

1.7 Statistical Analysis

Original data were organized and statistically analyzed using Excel 2010, Turkey' s test was performed using SPSS 21.0, and figures were created using Origin 2021.

Results

2.1 Soil Microbial Biomass of *P. macrophyllus* Under Different NPK Addition Levels

As shown in Figure 1 [Figure 1: see original paper], nutrient addition treatments had significant effects on soil MBC, MBN, and MBC/MBN, with all nutrient addition treatments being significantly higher than the control (CK) treatment. Figure 2 [Figure 2: see original paper] shows that the abundance of soil microorganisms in *P. macrophyllus* soil decreased in the order: bacteria > actinomycetes > fungi > nitrogen-fixing bacteria, reaching orders of magnitude of 10^7, 10^6, 10^4, and 10^3, respectively. Bacteria were the dominant microbial group in *P. macrophyllus* soil, accounting for over 80% of the total soil microbial population, followed by actinomycetes at approximately 15%-20%, while fungi and nitrogen-fixing bacteria accounted for relatively small proportions of only a few thousandths. Bacterial and actinomycete numbers showed little difference under different NPK nutrient level additions, while fungal and nitrogen-fixing bacterial numbers were significantly higher under different NPK nutrient level additions compared to the control treatment. As shown in Figure 3 [Figure 3: see original paper], the T-value for P addition was the largest in the soil MBC index, indicating that soil MBC was most affected by P addition (the same applies below), with N addition and K addition having comparable influence. MBN was most affected by soil N addition and K addition, with P addition having the smallest effect. N addition had the greatest effect on bacteria, followed by P addition, with K addition having the smallest effect. N addition had the greatest effect on actinomycetes, fungi, and actinomycetes, while the effects of P addition and K addition were minor and negligible. Overall, *P. macrophyllus* soil MBC and MBN were regulated by different nutrients, with P addition being the main factor affecting MBC, while N addition and K addition were the main factors affecting MBN.

Different capital letters indicate different experimental treatments, and different lowercase letters indicate differences between treatments ($P < 0.05$). The same below.

2.2 Microbial Diversity Indices of *P. macrophyllus* Soil Under Different NPK Addition Levels

As shown in Table 3 , compared with the control, NPK addition significantly increased the Shannon index, Simpson index, McIntosh index, and Pielou index of *P. macrophyllus* soil microorganisms. Figure 4 [Figure 4: see original paper] shows that among the four microbial diversity indices, different nutrient additions had varying degrees of influence. N, P, and K additions all had relatively large effects on the Shannon index, with the largest T-values for N addition and P addition and the smallest for K addition. For the Simpson index and McIntosh index, N addition had the largest T-value, while P addition and K addition had smaller T-values that were negligible. For the Pielou index, N, P, and K additions all had relatively large effects, with the largest T-value for N addition, followed by P addition, and then K addition.

2.3 Carbon Source Utilization Characteristics of *P. macrophyllus* Soil Microbial Communities Under Different NPK Addition Levels

As shown in Figure 5 [Figure 5: see original paper], the AWCD of nutrient levels entered a rapid growth phase after 24 h of cultivation and gradually stabilized after 120 h when the carbon sources in the Biology microplate were consumed. Treatment A showed significantly higher utilization of five types of soil microbial carbon sources than other treatments. The Biology microplate contains 96 microwells with 31 carbon sources, which were classified into six categories based on chemical group properties: amines, carboxylic acids (CAA), polymers, carbohydrates, amino acids (AMA), and other compounds (OC). As shown in Figure 6 [Figure 6: see original paper], the microbial community under treatment A soil nutrient level showed high utilization of all carbon sources. Carbohydrates, amino acids, carboxylic acids, and other compounds showed the lowest utilization characteristics at treatment I. The control treatment showed the lowest utilization of polymer and amine carbon sources but moderate utilization of other carbon sources.

2.4 Height and Ground Diameter Increment of *P. macrophyllus* Seedlings Under Different NPK Addition Levels

As shown in Table 4 , the trends in height and ground diameter increment of *P. macrophyllus* in response to soil nutrient levels were consistent with the response trends of microbial community functional diversity. Nutrient addition treatments were higher than the control treatment. N addition had the greatest effect on *P. macrophyllus* height growth, followed by P addition, with K addition having the smallest effect. N addition had the greatest effect on ground diameter growth, followed by K addition, with P addition having the smallest effect.

2.5 Analysis of NPK Addition Effects on *P. macrophyllus* Soil Microbial Community Functional Diversity

Redundancy analysis (RDA, Figure 7 [Figure 7: see original paper]) and CCA heatmap (Figure 8 [Figure 8: see original paper]) revealed the effects of NPK addition on soil microbial community functional diversity. The results showed that nutrient addition had significant effects on *P. macrophyllus* soil microbial community functional diversity, with N addition being the dominant factor. *P. macrophyllus* soil fungal abundance ($P < 0.001$), nitrogen-fixing bacterial abundance ($P < 0.01$), Simpson index ($P < 0.05$), and McIntosh index ($P < 0.001$) were all significantly positively correlated with N addition, while bacterial abundance ($P < 0.05$), actinomycete abundance ($P < 0.001$), AWCD ($P < 0.001$), Pielou index ($P < 0.001$), MBC/N ($P < 0.05$), amine carbon source utilization intensity ($P < 0.001$), carboxylic acid carbon source utilization intensity ($P < 0.001$), polymer carbon source utilization intensity ($P < 0.001$), and other compound carbon source utilization intensity ($P < 0.001$) were all significantly negatively correlated with N addition. The Shannon index ($P < 0.05$) was significantly negatively correlated with P addition. The Shannon index ($P < 0.05$), Pielou index ($P < 0.05$), amino acid carbon source utilization intensity ($P < 0.01$), and carbohydrate carbon source utilization intensity ($P < 0.001$) were significantly negatively correlated with K addition. *P. macrophyllus* height increment and ground diameter increment were negatively correlated with N addition and positively correlated with K addition, but neither reached significant levels, while both height increment ($P < 0.001$) and ground diameter increment ($P < 0.01$) were significantly positively correlated with P addition. The CCA heatmap (Figure 8) showed varying degrees of correlation among microbial functional diversity indices. Bacterial and actinomycete abundance and microbial community richness and evenness were positively correlated with the utilization intensity of six carbon sources to varying degrees, while fungal and nitrogen-fixing bacterial abundance and microbial community consistency and dominance were negatively correlated with the utilization intensity of six carbon sources to varying degrees.

Discussion

3.1 Effects of NPK Addition on *P. macrophyllus* Soil Microbial Biomass and Diversity

In this study, bacteria were the dominant microbial group in *P. macrophyllus* soil, accounting for over 80% of the total soil microbial population, followed by actinomycetes at approximately 15%-20%, while fungi and nitrogen-fixing bacteria accounted for relatively small proportions of only a few thousandths, consistent with previous research conclusions (Zhang, 2018). In this study, nutrient addition, especially N addition, significantly increased fungal abundance, which may be related to fungal adaptability. Research has shown that fungi are better adapted to environments with NPK addition (Zhou et al., 2016; Fang et al., 2019). Additionally, N addition increases plant root or soil microbial demand for P, leading to elevated soil phosphatase activity, which indirectly increases fungal abundance (Norisada et al., 2006; Ushio et al., 2010). *P. macrophyllus* soil contained considerable nitrogen-fixing bacterial populations. Many studies have shown that soil N addition causes nitrogen-fixing bacteria to compete with other microorganisms, thereby reducing their abundance (Li et al., 2019). However, in this study, N addition significantly increased nitrogen-fixing bacterial abundance. Sun et al. (2022) demonstrated that N addition can promote nitrogen-fixing bacteria in nodulating root systems that do not have nitrogen-fixing functions, and the large number of root nodules present in *P. macrophyllus* roots may be the reason for the increase in nitrogen-fixing bacteria with N addition. The nitrogen-fixing function of these bacteria can increase soil available nitrogen content (Huang et al., 2014), forming a positive feedback relationship with soil N addition that further strengthens the impact of N addition on soil microbial functional diversity. In this study, high nutrient addition reduced bacterial and actinomycete abundance, but the reduction was small, indicating that nutrient addition had minor effects on bacteria and actinomycetes, a result consistent with previous studies (Li et al., 2019). The carbon-to-nitrogen ratio is an important factor affecting soil microbial structure (Williamson et al., 2005), suggesting that under constant soil organic carbon, nitrogen addition amount is the main factor influencing soil microbial structure.

The Shannon index reflects microbial community richness, the Simpson index reflects dominance, the McIntosh index represents a diversity index based on multidimensional spatial distance of community species (essentially microbial community consistency), and the Pielou index indicates evenness. In this study, microbial community diversity was mainly affected by N. Combined with the inhibitory effects of N addition on bacteria and actinomycetes and its promotional effects on fungi and nitrogen-fixing bacteria, N addition may have altered soil organic matter composition in the absence of organic nutrient addition, thereby changing organic matter flow in the soil food chain and affecting microbial metabolic activity (Su, 2015). Increased N addition may reduce or inhibit the abundance and diversity of highly sensitive microorganisms in *P. macrophyllus* soil (Wang et al., 2018) while making dominant microbial groups more dominant and increasing consistency. For example, soil saprotrophic fungi, especially basidiomycetes, are positively correlated with nutrient addition (Wang et al., 2017), and due to increased competitiveness and abundance of these fungi, other microbial species grow slowly or even disappear. Over time, soil microbial diversity declines severely and the microbial community becomes singular, which is unfavorable for *P. macrophyllus* growth and development.

3.2 Effects of NPK Addition on Carbon Source Utilization Characteristics and Functional Diversity of *P. macrophyllus* Soil Microorganisms

Different NPK addition rates significantly affected carbon source utilization intensity by *P. macrophyllus* soil microorganisms, and the ability of soil microbial communities to utilize different carbon sources reflects changes in soil microbial community functional diversity (Tian & Wang, 2011). In this study, the intensity of carbon source utilization by soil microorganisms was significantly correlated with nutrient addition and soil microbial diversity, similar to previous research (Su et al., 2022). Different nutrient additions have different effects on *P. macrophyllus* seedlings, thereby affecting the types and quantities of root exudates. In this study, the utilization intensity of amino acids and carbohydrates with NPK addition was significantly higher than in the control treatment, indicating that amino acids and carbohydrates are the main root exudates of *P. macrophyllus*. These exudates are carbon sources that are relatively easy for soil microorganisms to utilize and show the greatest utilization intensity (Chapin et al., 1993; Zhang et al., 2020). After being transformed by microorganisms, they synthesize plant growth regulators that can be absorbed and utilized by plants, promoting plant growth (Li, 2021). Amines, carboxylic acids, polymers, and other compounds are types of soil carbon sources affected by specific microorganisms (Hiraishi & Khan, 2003; Zhalnina et al., 2018; Zhang et al., 2020). The utilization intensity of these difficult-to-use carbon sources significantly decreased with increasing nutrient addition, possibly because increased N, P, and K addition raised soil salt concentration (Zhou et al., 2017), thereby significantly reducing carbon source utilization intensity by soil microorganisms (Wang et al., 2020; Wang, 2022) and directly decreasing soil microbial carbon source utilization intensity. This study also showed that N addition indirectly reduced the utilization intensity of difficult-to-use carbon sources by soil microbial communities by decreasing bacterial and actinomycete abundance, increasing fungal and actinomycete abundance, reducing microbial community richness and evenness, and increasing microbial community consistency and dominance (Figure 8), leading to declines in *P. macrophyllus* soil quality and soil ecosystem stability that are unfavorable for seedling growth and development. In this study, *P. macrophyllus* height increment and ground diameter increment were significantly positively correlated with P addition, demonstrating the preference of potted *P. macrophyllus* for P element. The L9 range analysis and correlation analysis conclusions in this paper were relatively consistent and highly credible. However, this experiment was a pot trial using two-year-old *P. macrophyllus* seedlings with an experimental duration of only four months, which has certain limitations and is applicable to artificial cultivation environments. Whether similar conclusions can be obtained in field conditions requires further investigation.

Different nutrient addition levels had significant effects on soil microbial functional diversity. Overall, nutrient addition generally resulted in higher soil microbial community functional diversity and growth in *P. macrophyllus* than the control group, indicating that *P. macrophyllus* growth is generally fertilizer-responsive. However, different indices showed different responses to varying addition rates of each element. N addition was the dominant factor affecting these changes. In summary, increased N addition significantly reduced bacterial and actinomycete abundance, microbial community richness and evenness, increased fungal and nitrogen-fixing bacterial abundance, and increased microbial community dominance and consistency, thereby reducing the utilization of difficult-to-use carbon sources by *P. macrophyllus* soil microorganisms. The positive feedback effect between nitrogen-fixing bacteria and nitrogen content deepened this impact. Increased P addition significantly reduced soil microbial community richness. Increased K addition significantly reduced soil microbial community richness and evenness and the utilization intensity of easily available carbon sources by microbial communities. Therefore, fertilization management is still necessary for *P. macrophyllus* cultivation, but attention should be paid to applying fertilizer in small amounts and multiple times, appropriately reducing N and K addition rates and increasing P addition to create a favorable soil ecological environment for *P. macrophyllus* growth and promote its sustainable cultivation.

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Note: Figure translations are in progress. See original paper for figures.