

Isolation and Identification of Rhizosphere Microorganisms and Endophytes in *Pogostemon cablin*

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Abstract [Objectives] To systematically investigate the microbial community composition of rhizosphere soil and endophytes associated with *Pogostemon cablin*, and to explore the relationships between endophytes and rhizosphere microorganisms as well as their potential applications. [Methods] Microbial isolates were obtained from rhizosphere soil, root tissues, and stem tissues using the serial dilution and spread plate method. These isolates were identified through morphological characterization, physiological and biochemical assays, and molecular biological techniques. [Results] A total of 18 microbial strains were isolated, including 7 bacterial and 11 fungal strains. Among the bacterial isolates, *Pseudomonas* spp. and *Bacillus* spp. were predominant, while the fungal isolates were mainly represented by *Aspergillus* spp. Certain bacterial strains, notably *Pseudomonas* spp., exhibited potential abilities for indole-3-acetic acid (IAA) production, nitrogen fixation, and antagonistic activity against pathogenic microorganisms, suggesting their potential utility as biocontrol agents and promoters of plant growth. [Conclusions] This study establishes a foundational understanding of the microbial community characteristics in the rhizosphere and tissues of *P. cablin*, as well as their roles in plant growth and development.

Key words *Pogostemon cablin*, Rhizosphere microorganism, Endophyte, Separation and identification

1 Introduction

Rhizosphere microorganisms and plant endophytes are intimately associated with plant growth and development, playing a crucial role in maintaining plant health. Notably, rhizosphere microorganisms are often referred to as the "second genome" of plants^[1]. Studies have demonstrated that beneficial microorganisms in the rhizosphere can mitigate damage to plants caused by natural environmental stressors, stabilize the structure of soil microbial communities, secrete plant hormones that promote growth, and effectively antagonize pathogenic bacteria, thereby enhancing plant stress resistance^[2–3]. Endophytes are ubiquitously present in plants and have established stable mutualistic symbiotic relationships with their host plants through long-term co-evolution. Research indicates that endophytes can regulate plant hormones, stimulate root development, enhance nutrient absorption, confer resistance to environmental stresses and diseases, and promote overall healthy plant growth^[4]. Conversely, plants supply the essential nutrients and habitats required for the survival of endophytes. Certain endophytes contribute to plant growth by enhancing the root system or by facilitating nutrient transport in the soil^[5–6].

Pogostemon cablin is a traditional and authentic medicinal

herb native to the Lingnan region, frequently utilized as a raw material in Chinese patent medicines, including Huoxiang Zhengqi Pills. Its active constituents, such as pogostone and patchouli alcohol, exhibit a range of biological activities, including antibacterial, antioxidant, antiviral, and antitumor effects^[7–9]. This study isolated and identified microorganisms from the rhizosphere soil, root tissue, and stem tissue of *P. cablin*, thereby enriching the foundational data on endophytes and rhizosphere microbial resources associated with this species. The findings provide a theoretical basis for the research and development of microbial fertilizers derived from the microbial resources of *P. cablin*.

2 Materials and methods

2.1 Materials The rhizosphere soil, root tissue, and stem tissue of *P. cablin* were collected at the herb greenhouse of Zhongkai University of Agriculture and Engineering (Baiyun Campus) located at 113°26' E, 23°22' N. Three healthy *P. cablin* plants, free from diseases and pests, were randomly selected and carefully excavated along with their entire root systems. Rhizosphere soil, as well as corresponding root and stem tissues, were collected and placed separately in sterile bags, then stored at 4 °C for subsequent analysis.

2.2 Main culture media and reagents The materials utilized in this study included beef extract peptone medium, potato glucose agar medium (PDA), Gauze's synthetic broth medium, 5% sodium hypochlorite (NaClO) solution, 1% streptomycin solution, 0.85% sodium chloride (NaCl) solution, 0.25 mol/L sodium hydroxide (NaOH) solution, 3% hydrogen peroxide (H₂O₂) solution, Gram staining reagents, methyl red indicator, bacterial DNA extraction kit, 20% sodium dodecyl sulfate (SDS) lysate, and

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enzymes related to PCR amplification.

2.3 Methods

2.3.1 Isolation of rhizosphere soil microorganisms. Soil microor-ganisms from the rhizosphere were isolated using the serial dilution and spread plate techniques. Soil samples were prepared into sus-pensions with varying concentration gradients (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6}). For each concentration gradient, three replicates were prepared and inoculated onto beef extract peptone medium, PDA medium, and Gauze’s synthetic broth medium, re-spectively. The incubation temperature was set at 37 °C for bacte-ria and 28 °C for fungi and actinomycetes.

2.3.2 Isolation of endophytes. The root and stem tissues of *P. cablin*, collected from the field, were washed with deionized water, sectioned into 0.5 cm lengths, and dried using sterile filter paper. Subsequently, the samples were disinfected by immersion in 0.05% sodium hypochlorite solution and 75% ethanol for 2 min each. Following disinfection, the tissues were rinsed three times with sterile water to ensure complete removal of the disinfectants and were finally dried with sterile filter paper. The isolation of en-dophytes was conducted following the method described by Zhao Juan *et al.* [10]. Single-colony bacteria obtained through isolation and purification were cultured in a shaking incubator, preserved with glycerol, and stored at −80 °C.

2.3.3 Microbial morphological characteristics and physiological and biochemical experiments. Microbial single colonies were ini-tially classified based on their morphological characteristics and subsequently identified following the guidelines provided in *Bergey’s Manual of Determinative Bacteriology* [11] and *Microbial Taxonomy* [12]. Bacterial identification experiments encompassed the Gram staining test, string test, puncture test, catalase test, and methyl red test. The identification of fungi primarily relied on their morphological characteristics, including colony features (such as shape and color), mycelial features (such as branching patterns and the presence or absence of septa), and the location and arrangement of spores.

2.3.4 Molecular biological identification. Bacterial DNA was extracted utilizing the bacterial DNA extraction kit (Nanjing Vazyme Biotech Co. , Ltd.) following the manufacturer’s protocol. The 16S rDNA sequence was amplified using universal primers 27F (AGRGTTTGATYNTGGCTCAG) and 1492R (TASGGHTA-CCTGTTASGACTT), yielding amplified fragments of approxi-mately 1 500 bp. The extraction of fungal DNA was conducted using the SDS alkaline lysis method. Amplification of the fungal 18S rDNA sequence was performed employing the primers EukF (AACCTGGTTGATCCTGCCAGT) and EukR (GATCCTTCTG-CAGGTTACCTAC), yielding amplified fragments of approxi-mately 1 800 bp. Sequencing of the PCR products was carried out by Beijing Rui Biotech Co. , Ltd. , and the resulting sequences were analyzed by comparison with the NCBI database using BLAST.

3 Results and analysis

3.1 Isolation and purification of microorganisms Microor-ganisms from the rhizosphere soil, root, and stem tissues of *P. ca-blin* were isolated and purified, and the resulting single colonies were preliminarily classified (Table 1). Regarding sample types, a greater diversity of microorganisms was observed in the rhi-zosphere soil compared to the root and stem tissues, with the micro-bial types isolated from the root and stem tissues being identical. In terms of microbial groups, fungi exhibited the highest species diversity, followed by bacteria, whereas no actinomycetes were detected.

Table 1 Isolation and purification of rhizosphere microorganisms and endophytes in *Pogostemon cablin*

Sample name	Microbial type	Colony number CFU/g	Species number	Strain code
Rhizosphere soil	Bacteria	5.0×10^7	3	SX1, SX3, SX7
	Fungi	1.9×10^4	7	SZ1-4, SF1-3
	Actinomycetes	0	–	–
Root tissue	Bacteria	3.4×10^5	4	X2, X4-6
	Fungi	3.0×10^3	4	Z5-8
	Actinomycetes	0	–	–
Stem tissue	Bacteria	3.7×10^3	4	X2, X4-6
	Fungi	6.6×10^2	4	Z5-8
	Actinomycetes	0	–	–

NOTE " – " denotes the absence of this column.

3.2 Morphological characteristics and physiological and bio-chemical identification results of bacteria Following morpho-logical examination and physiological and biochemical character-ization of the isolated bacteria, the results are presented in Fig. 1. Strain SX1 was identified as belonging to the genus *Agrobacterium*; strains X2, SX3, X4, and X7 were classified within the genus *Ba-cillus*; strain X5 was assigned to the genus *Pseudomonas*; and strain X6 was identified as a member of the genus *Acinetobacter*.

The results of physiological and biochemical experiments demonstrated that all isolated bacterial strains exhibited catalase activity. The observation that strains X3, X5, and X7 dispersed in the liquid beef extract medium indicated their motility via flagella. Additionally, strains X2, SX3, and X6 possessed the capability to metabolize glucose (Table 2).

3.3 Morphological identification results of fungi The mor-phological characteristics of the 11 isolated fungal strains were ex-aminated, and the results are presented in Fig. 2. Strain SZ1 exhib-ited a moderately rapid growth rate, forming flat colonies with well-defined edges. The mycelium appeared white and possessed a vel-vety texture. Microscopic observation revealed clustered conidia, with spores that were spherical to nearly spherical in shape, featur-ing a smooth surface and a high abundance of large, white conidia. Strain SZ2 exhibited relatively slow growth, forming flat colonies characterized by radiating wrinkles. The mycelium’s outer edge was white, whereas the center displayed an earthy yellow col-oration. Additionally, the conidiophores possessed a comparatively thick diameter. Strain SZ3 exhibited slow growth, with colonies displaying a semi-villous surface and white coloration. Microscopic

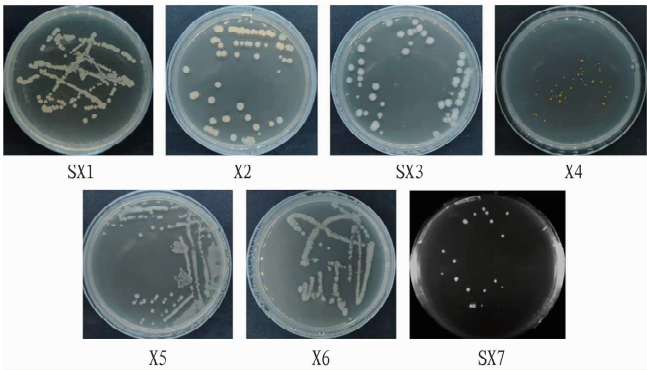


Fig. 1 Images of purified colonies of different bacteria

Table 2 Morphological characteristics and physiological and biochemical identification results of different bacteria

Strain code	Bacterial string	Gram staining	Catalase	Methyl red	Morphology	Colony characteristics
SX1	G ⁻	G ⁻	+	-	Rod-shaped	Round, light yellow, with smooth and lustrous surface, and neat edges
X2	G ⁺	G ⁺	+	+	Rod-shaped	Round, raised, light yellow, opaque, with neat edges
SX3	G ⁺	G ⁺	±	+	Rod-shaped	Round, milky white, raised, opaque, with uneven edges
X4	G ⁺	G ⁺	+	-	Rod-shaped	Round, yellow, with a raised and opaque center, and uneven edges
X5	G ⁻	G ⁻	+	-	Rod-shaped	Round, milky white, with smooth, moist, and viscous surface
X6	G ⁻	G ⁻	+	+	Rod-shaped	Round, milky white, opaque, with smooth and lustrous surface
SX7	G ⁺	G ⁺	±	-	Rod-shaped	Round, white, opaque, with smooth and lustrous surface

NOTE S. Rhizosphere; G⁺. Gram-positive bacteria; G⁻. Gram-negative bacteria; +. Positive; -. Negative.

slight radiating patterns and a shriveled morphology, bordered by well-defined edges. The conidia were nearly spherical to ellipsoidal, colorless, and produced short spore chains that were loosely arranged. Strain Z6 exhibited slow growth, forming colonies that were cotton-like and soft in texture, with well-defined edges, uniform expansion, and closely adhered to the surface of the culture medium. The colonies appeared white, and microscopic examination revealed slender, branched mycelia. Strain Z7 exhibited rapid growth, forming colonies that were cotton-like and white in appearance. Microscopic examination revealed that the mycelium was slender and branched. The growth rate of strain Z8 was moderately rapid. The mycelium exhibited a yellowish-brown coloration, with a velvety surface texture and a loose texture. The colony margins were well-defined and displayed slightly radial patterns. The mycelium produced melanin, resulting in the blackening of the culture medium. Microscopic examination revealed that the mycelium was slender and branched. The growth rate of strain SF1 was relatively slow, forming colonies with a white leading edge and a villous surface. The strain produced pigment but did not generate spores. Microscopic examination revealed that the mycelia were highly branched. Strain SF2 exhibited rapid growth. The initial colonies appeared white and fluffy, subsequently transitioning to an olive green hue. The colony surface was loose and wool-like, with well-defined edges. Microscopic examination revealed spores that were characteristically lemon-shaped with a smooth surface. Upon maturation, the spores became light brown and developed micro-protrusions at both poles. The colonies of strain SF3 exhibited rapid growth and were initially white and fluffy, characterized by a dry surface, loose texture, and well-defined edges displaying radiating stripes. Microscopic examination revealed transparent, branched mycelia and upright conidiophores with a typical phi-

examination revealed that the mycelium was of a single mycelial type, transparent and smooth, with no conidia or other asexual reproductive structures observed. The growth rate of strain SZ4 was comparatively slow, with colonies exhibiting a light grayish-green coloration, a loose texture, and a velvety appearance. The colony surface exhibited a slightly radiating and wrinkled texture, characterized by well-defined edges. Microscopic examination revealed conidia arranged in clusters. The spores were spherical with smooth surfaces. The quantity of conidia was limited, and they displayed a light green hue. Strain Z5 exhibited slow growth, forming colonies that were light yellowish-brown in color, with a loose texture and velvety appearance. The colony surfaces displayed

alide-shaped structure. The conidia were spherical to ellipsoidal, possessed smooth surfaces, and were arranged in short chains or small clusters.

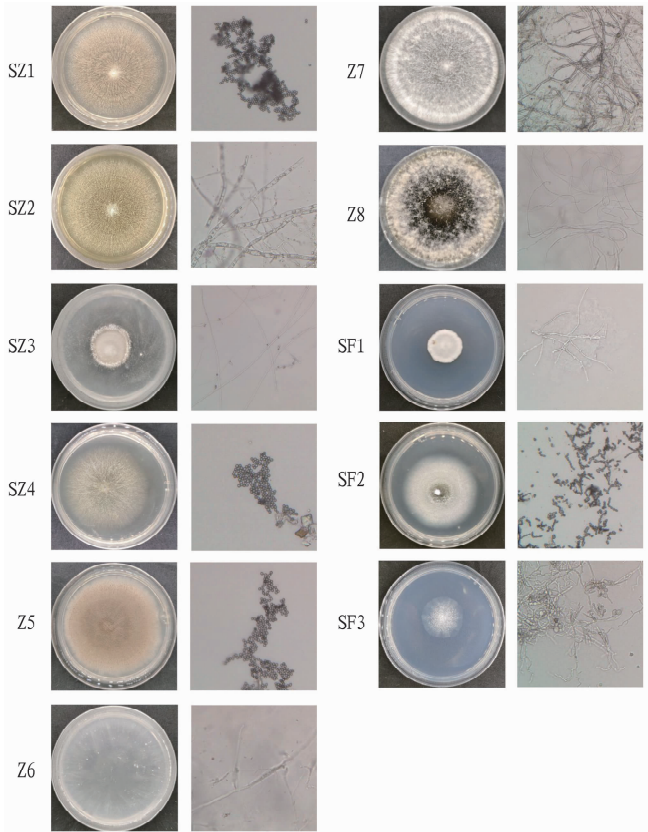


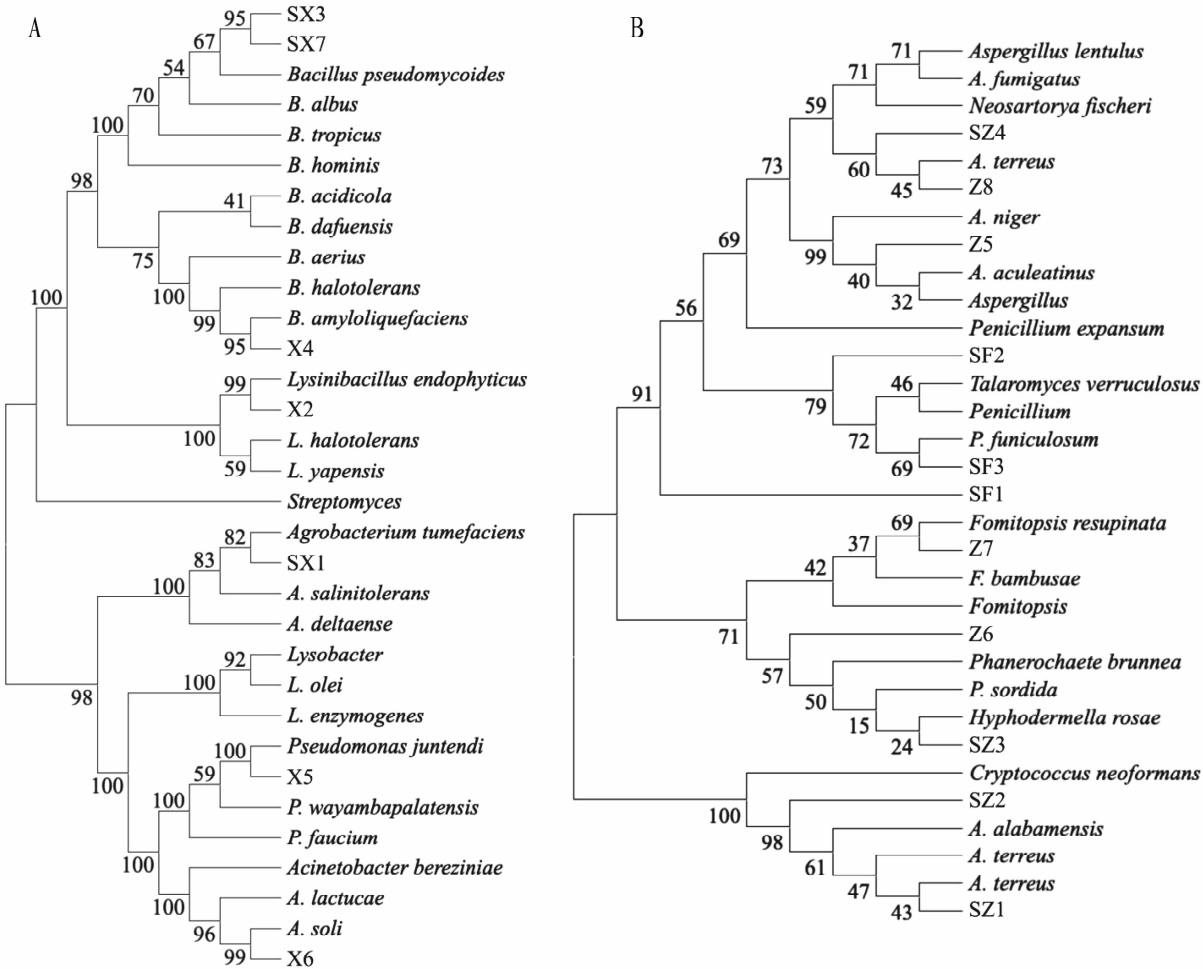
Fig. 2 Images of purified colonies of different fungal isolates

3.4 Molecular biology identification results The 18 isolated microorganisms underwent molecular identification through analysis of 16S rDNA and 18S rDNA sequences. Homology alignment was performed by comparing the obtained sequences with those of related species available in GenBank. The results of this comparison are presented in Table 3.

The 7 bacterial species identified were *Agrobacterium tumefaciens*, *Lysinibacillus endophyticus*, *Bacillus tropicus*, *Bacillus amyloliquefaciens*, *Pseudomonas juntendi*, *Acinetobacter soli*, and *Bacillus albus*. Additionally, 11 fungal species were identified, including *Aspergillus alabamensis*, *Aspergillus terreus*, *Hyphodermella rosae*, *Aspergillus lentulus*, *Aspergillus aculeatinus*, *Phanerochaete* spp., *Fomitopsis resupinata*, *Aspergillus fischeri*, *Penicillium corylophilum*, *Talaromyces verruculosus*, and *Talaromyces funiculosus*. Phylogenetic trees for bacteria and fungi were constructed separately using MEGA11 software employing the neighbor-joining method (the confidence levels were assessed through 1 000 bootstrap replicates). The results are presented in Fig. 3, where panel A depicts the bacterial phylogenetic tree and panel B illustrates the fungal phylogenetic tree.

Table 3 Sequence comparison results of 18 types of microorganisms

No.	Strain code	Strain with the highest homology	Homology %	Source
1	SX1	<i>Agrobacterium tumefaciens</i>	99.89	NR_116306.1
2	X2	<i>Lysinibacillus endophyticus</i>	99.55	NR_146821.1
3	SX3	<i>Bacillus tropicus</i>	99.89	NR_157736.1
4	X4	<i>Bacillus amyloliquefaciens</i>	98.87	NR_041455.1
5	X5	<i>Pseudomonas juntendi</i>	99.32	NR_116172.1
6	X6	<i>Acinetobacter soli</i>	100.00	NR_044454.1
7	SX7	<i>Bacillus albus</i>	99.32	NR_157729.1
8	SZ1	<i>Aspergillus alabamensis</i>	99.49	NR_166027.1
9	SZ2	<i>Aspergillus terreus</i>	99.83	NR_131276.1
10	SZ3	<i>Hyphodermella rosae</i>	98.28	JN940190.1
11	SZ4	<i>Aspergillus lentulus</i>	99.66	XR_004500618.1
12	Z5	<i>Aspergillus aculeatinus</i>	99.89	MK235149.1
13	Z6	<i>Phanerochaete</i> spp.	88.25	GU190189.1
14	Z7	<i>Fomitopsis resupinata</i>	98.00	OL621768.1
15	Z8	<i>Aspergillus fischeri</i>	99.66	U21299.1
16	SF1	<i>Penicillium corylophilum</i>	99.61	KT582518.1
17	SF2	<i>Talaromyces verruculosus</i>	99.43	KT897711.1
18	SF3	<i>Talaromyces funiculosus</i>	97.68	JN938961.1



NOTE A. Phylogenetic analysis of bacteria; B. Phylogenetic analysis of fungi.
Fig.3 Phylogenetic analysis of bacteria and fungi

4 Conclusions and discussion

In this study, microbial strains were isolated from the rhizosphere soil, root tissue, and stem tissue of *P. cablin* and systematically identified through traditional morphological observations, physiological and biochemical assays, and molecular biological techniques. The results indicated that the identification outcomes derived from morphological and physiological biochemical assays were largely consistent with those obtained through molecular biological techniques. However, traditional identification methods often present several limitations, such as complex experimental procedures, significant subjectivity in their application, and limited resolution, frequently resulting in accuracy restricted to the genus level. Molecular biology techniques offer several advantages, including high throughput, rapid processing, operational simplicity, and high accuracy in identification. These methods enable strain identification at a species level.

P. cablin harbors a diverse community of rhizosphere microorganisms and endophytes resources. Several microorganisms isolated in this study exhibit potential plant growth-promoting and antibacterial properties, indicating their beneficial roles. Among the isolates, the *Pseudomonas* strain identified was consistent with the genus *Pseudomonas* spp. previously isolated by Shi Wenguang *et al.*^[13] from *P. cablin*. This strain exhibited both the 3-OH PAME quenching enzyme gene and antagonistic activity against bacterial wilt. Growth characteristic analysis demonstrated that the strain possesses the ability to secrete indoleacetic acid (IAA), siderophores, proteases, organic acids, and other metabolites. Studies have demonstrated that *Lysinibacillus endophyticus* is capable of producing IAA, which promotes plant growth^[14]. *Bacillus* species, identical to those isolated from tomatoes by Guo Haoqun *et al.*^[15], have been shown to inhibit the growth of *Ralstonia solanacearum*. Li Yanyan *et al.*^[16] reported that *Aspergillus* exhibited a relatively high abundance in disease-suppressive soils against tobacco bacterial wilt, and its relative abundance was significantly negatively correlated with the incidence of the disease. Xie Huarong *et al.*^[17] isolated a species of the genus *Talaromyces* employing a double-layer plate antagonism method, which demonstrated a relative control efficacy of 57.47% against *R. solanacearum*. In conclusion, the roots of *P. cablin* harbor a diverse array of microbial resources. Further comprehensive studies can be undertaken to investigate the biological characteristics, growth-promoting effects, and antibacterial properties of these beneficial microorganisms, thereby offering a foundational reference for the development of microbial agents.

This experimental study initially characterized the population composition and diversity of rhizosphere microorganisms and endophytes associated with *P. cablin*. Significant differences were observed among bacterial and fungal genera and their respective abundant groups. Certain strains demonstrated potential application value, offering theoretical support for their development and

utilization in agricultural microecological formulations and plant growth promoters.

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