



The Composition and Phosphate-Solubilizing Capability of Phosphate-Solubilizing Bacteria in the Rhizosphere of Wild *Camellia oleifera* in Mountain Lushan

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Abstract: *Camellia oleifera*, a unique edible oil tree species in China, is of important economic value. However, the shortage of phosphorus in the soil is one of the important factors limiting the growth of *C. oleifera*. Here, we investigated the population size and composition of culturable phosphate-solubilizing bacteria (PSB) in the rhizosphere soil of wild *C. oleifera* in Mountain Lushan, China. PSB were isolated using a dilution coating plate method and identified by 16S rRNA sequencing. The phosphate-solubilizing capability of the isolated PSB was evaluated by a semi-quantitative method (the ratio of phosphate solubilization halo diameter versus colony diameter). The results showed that large amounts of PSB existed in the rhizosphere soil of wild *C. oleifera* ($0.28\text{--}1.08 \times 10^7$ CFU/g soil) and the population size of PSB differed from investigated trees. A total of 100 strains of PSB were isolated from the rhizosphere soil, belonging to *Bacillus*, *Burkholderia*, *Pantoea*, *Paraburkholderia*, and *Pseudomonas*, respectively. Of these strains, *Burkholderia* showed the highest isolation frequency and phosphate-solubilizing capability, accounting for 61% of the isolates. The phosphate solubilization index of 100 strains varied from 1.02 to 3.04 after a 6-day incubation, and *Bacillus* strains were easy to lose their phosphate-solubilizing capability during the incubation. Our result suggested that *Burkholderia* was the dominant genus of PSB in the rhizosphere of *C. oleifera* and could be utilized for facilitating the uptake of P.

Keywords: *Camellia oleifera*, Phosphate-solubilizing Bacteria, 16S rRNA, Phosphate Solubilization Index

1. Introduction

Phosphorus (P) is one of the most important macronutrients for the basic metabolic and physiological processes in plants including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis, and respiration, etc. [1]. Although the total P content is high in soils, the majority is in insoluble forms difficult to be absorbed by plants. Soluble

inorganic phosphate (Pi) is the only form available to plants directly, but its concentration in the soil is very low, usually 1–10 μM , only accounting for about 5% of total P in the soil [2].

Camellia oleifera (Theaceae), a unique edible oil tree species in China, is one of the world's famous woody oil plants [3]. Tea oil obtained from the seeds has oleic acid and linoleic acid-based unsaturated fatty acid contents up to 90%, along with large quantities of vitamin E, squalene, and flavonoid substances that are reported to have good health

care effect on digestion, immunity, reproduction, heart and cerebrovascular, etc., known as “Oriental olive oil” [4]. However, available Pi content in the soil of *C. oleifera* is often in a state of shortage, which is an important factor limiting the growth of *C. oleifera* [5, 6]. There are a large number of microorganisms in the soil, which can transform P resource that is difficultly accessed by plants into a form that can be absorbed and utilized. Solubilization of insoluble Pi by microorganisms was firstly reported by Pikovskaya [7]. Phosphate-solubilizing microorganisms are important microbial resources closely related to plant nutrition, accounting for about 10% of the total soil microorganisms [8]. At present, 44 genera of phosphate-solubilizing bacteria (PSB) have been reported, of which *Actinomyces*, *Bacillus*, *Burkholderia*, *Pseudomonas*, and *Streptomyces* are the most common taxa [9-14]. In recent years, studies showed that there are larges of PSB taxa in the rhizosphere of *C. oleifera* [14-17], suggesting the enrichment of PSB in the rhizosphere may be one of the important mechanisms for *C. oleifera* adapting to low P stress. For example, *Bacillus aryabhatai* JX285 and *Pseudomonas auricularis* HN038 isolated from the rhizosphere of *C. oleifera* can significantly increase the available P content in the rhizosphere and the total P content in *C. oleifera* trees [18]. The insoluble Pi in soils is mainly divided into two types: inorganic Pi and organic Pi, and the insoluble inorganic Pi is dominant [19]. However, previous studies on rhizospheric PSB of *C. oleifera* mainly focused on their biodegradation of organic phosphorus [15-17], but the phosphate-solubilizing capability on insoluble Pi by PSB and their taxonomic composition in the rhizosphere of *C. oleifera* are still unclear. Therefore, this study aimed to study the composition of PSB in the rhizosphere of *C. oleifera* based on a culture-dependent method and analyzed their differences in phosphate-solubilizing capability, which will be conducive to mining PSB resource to promote the growth of *C. oleifera*.

In this study, we determined the population size of PSB in the rhizosphere soil of wild *C. oleifera* in Mountain Lushan, Jiangxi Province, China. PSB were isolated using a plating method and identified by 16S rRNA sequencing. The phosphate-solubilizing capability of the isolated PSB was evaluated by a semi-quantitative method. We aimed to reveal the community composition of PSB in the rhizosphere soil of *C. oleifera* based on a culture-dependent method, assess their phosphate-solubilizing capability, and screen potential efficient PSB.

2. Materials and Methods

2.1. Study Site and Sample Collection

The study site is located in Mountain Lushan, Jiangxi province, China, where the annual average temperature is 11.4°C and the annual average rainfall is 1,916 mm. Rhizosphere soil samples of wild *C. oleifera* were collected at Sanbaoshu scenic area in September 2020. This area is an evergreen and deciduous broad-leaved mixed forest with an altitude of about 900 m. Five *C. oleifera* trees (CO1, CO2, CO3, CO4, and CO5) were chosen and each other was over 10

m apart. Roots of *C. oleifera* were collected and the soil attached to the root was taken as rhizosphere soil after removing bulk soil by slight shaking. Then, rhizosphere soil was obtained and collected by ultrasonic cleaning in sterilized physiological saline solution (0.85%) for 10 min and centrifuging at 10,000 rpm for 5 min.

2.2. Population Size of PSB in the Rhizosphere of *C. oleifera*

Five-gram rhizosphere soil was floated in 50ml sterilized physiological saline solution. An aliquot (100 µl) from 10⁴, 10⁵ and 10⁶ dilution was respectively inoculated on NBRIP agar media (Glucose 10 g, NaCl 0.2 g, KCl 0.2 g, (NH₄)₂SO₄ 0.5 g, MgSO₄·7H₂O 0.3 g, MnSO₄ 0.03 g, FeSO₄·7H₂O 0.01 g, tricalcium phosphate 5 g, agar 15 g, pH 7.0), and incubated at 30 °C for three days. Each dilution was in triplicate. After incubation, the colony-forming units (CFU) of bacteria with solubilization halos were counted.

2.3. Isolation, Purification and Identification of PSB

Colonies showing large clear halo zone of phosphate solubilization appearing on agar media were streaked on fresh NBRIP agar media for purification and finally transferred in NBRIP agar slants and stored at a 4 °C refrigerator for further studies. A total of 20 PSB strains were randomly selected from each rhizosphere of *C. oleifera*. The selected isolates were respectively incubated in Luria-Bertani (LB) liquid media at 30 °C and 180 rpm for two days for proliferation. Then, genomic DNA of isolates was extracted using Rapid Bacterial Genomic DNA Isolation Kit (Sangon Biotech, China) according to the manufacturer's instruction. The amplification of 16S rRNA referred to the method of Sun *et al.* [20]. The amplified products were sent for Sanger sequencing at Sangon Biotech, China. Sequences were analyzed by the BLAST algorithm for comparison of a nucleotide query sequence against GenBank of NCBI to find the closely related sequences. Phylogenetic relationships of selected PSB were constructed using the Maximum Likelihood method in MEGA 7.0 software [21].

2.4. Phosphate-solubilizing Capability Assay of PSB

Each strain was incubated in LB liquid medium for two days at 30 °C and 180 rpm. Then, the phosphate-solubilizing capability of PSB was evaluated after inoculating 4 µl of inocula on NBRIP agar media at 30 °C for six days. The growth and diameter of solubilization halos were measured from the 2nd day to the 6th day. The phosphate-solubilizing capability was evaluated by phosphate solubilization index (PSI) that was calculated according to the following formula [22]:

$$PSI = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}}$$

2.5. Statistical Analysis

All the results were statistically analyzed using IBM SPSS Statistics 20 Software. A comparison between treatments was

performed using one-way analysis of variance (ANOVA) identified by Tukey’s HSD test at $p < 0.05$. The heatmap was built using the *heatmap* package in R 3.6.3 software [23]. Data are expressed as mean \pm standard deviation.

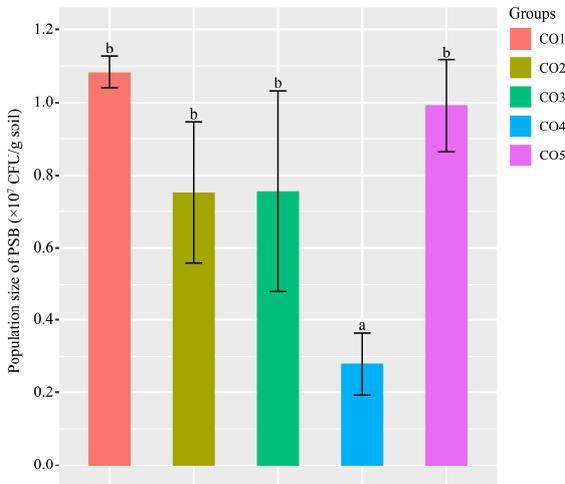


Figure 1. Population size of phosphate-solubilizing bacteria (PSB) in different rhizospheres of *C. oleifera*. CO1, CO2, CO3, CO4, and CO5 donate different rhizosphere soil samples of *C. oleifera*, respectively.

3. Results

3.1. Population Size of PSB in the Rhizosphere of *C. oleifera*

The population size of culturable PSB in the rhizosphere of

C. oleifera in Mountain Lushan was evaluated by counting the number of phosphate solubilization halos on NBRIP agar media. There was no notable difference in the population size of PSB in different *C. oleifera* rhizospheres. The number of PSB in rhizospheres of five *C. oleifera* trees was $0.77 \times 10^7 \pm 0.31 \times 10^7$ CFU/g soil. Except for sample CO4, the number of PSB in its rhizosphere was $0.28 \times 10^7 \pm 0.09 \times 10^7$ CFU/g soil, significantly lower than that in other rhizospheres ($p < 0.05$, Figure 1).

3.2. Taxonomic Composition and Phylogenetic Relationships of PSB

A total of 100 strains were isolated from the rhizosphere soil of five *C. oleifera* trees, belonging to *Burkholderia*, *Pantoea*, and *Pseudomonas* in Proteobacteria and *Bacillus* in Firmicutes. *Burkholderia* showed the highest isolation frequency (the number of *Burkholderia* strains versus the total number of isolated strains), accounting for 61% of the obtained isolates, followed by *Bacillus* (17%) and *Pseudomonas* (14%). Detailly, *Burkholderia* accounted for 80% of the strains isolated from the rhizosphere soil of CO1, 40% from CO2, 55% from CO3, 95% from CO4, and 35% from CO5, respectively. In addition, the result of phylogenetic analysis of the isolated *Burkholderia* strains showed that there was no significant difference in the genetic relationships among *Burkholderia* strains from different rhizosphere soils of *C. oleifera*, except that strains in CO5 could be clustered into two main clades (Clade 6 and Clade 11, Figure 2).

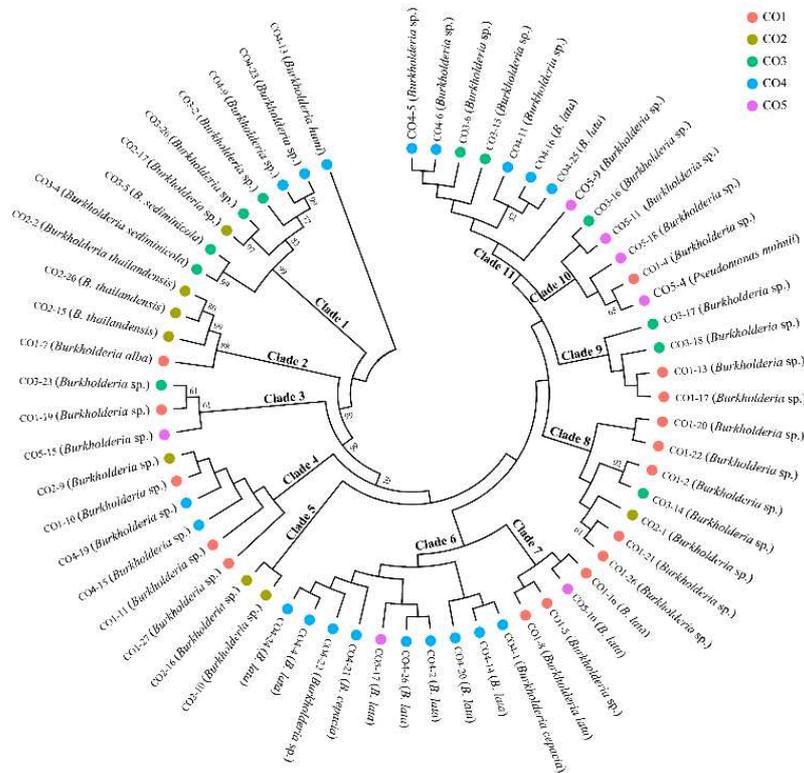


Figure 2. Phylogenetic tree of *Burkholderia* strains isolated from the rhizosphere of *C. oleifera* inferred from 16S rRNA sequences. Molecular phylogenetic analysis was using the Maximum Likelihood (ML) method based on the Tamura-Nei model conducted in MEGA7. The discrete Gamma distribution was used to model evolutionary rate differences among sites. Significant bootstrap support (>50) is indicated above branches.

3.3. Difference in the Phosphate-solubilizing Capability of PSB

A point inoculation method was used to compare the phosphate-solubilizing capability of PSB isolated different rhizospheres of *C. oleifera*. Figure 3 showed diverse colonial types and phosphate-solubilizing halos formed on NBRIP agar media by PSB isolated from rhizospheres of samples CO1. The values of PSI of the 100 strains from five different rhizospheres of *C. oleifera* were analyzed as shown in Figure 4. Most halo diameters were increased gradually in the period of incubation but differed from different strains. The top five strains with the strongest phosphate-solubilizing capability were CO1-5 (*Burkholderia* sp., PSI= 3.05 ± 0.13), CO5-11 (*Burkholderia* sp., PSI= 2.45 ± 0.05), CO1-2 (*Burkholderia* sp., PSI= 2.36 ± 0.09), CO1-13 (*Burkholderia* sp., PSI= 2.38 ± 0.26), CO5-9 (*Burkholderia* sp., PSI= 2.26 ± 0.02), respectively. The results also showed that six strains (*Bacillus* species) lost phosphate-solubilizing capability or form no halo during the incubation.

4. Discussion

Phosphorus is a vitally important mineral nutrient for the growth and reproduction of *C. oleifera*, but the available P content in the soil where *C. oleifera* trees grow is usually in deficient [5]. Fortunately, large amounts of beneficial rhizobacteria exist in soils, such as PSB that can promote the mobilization of insoluble Pi. In this study, we found many PSB existed in the rhizosphere soil of wild *C. oleifera* trees ($0.28\text{--}1.08 \times 10^7$ CFU/g soil, Figure 1), suggesting that may play an important role in the plant against P deficiency. A total of 100 strains of PSB were isolated from the rhizosphere soil using NIRBP agar media. The strains were identified by 16S rRNA sequencing and belonged to four genera (*Bacillus*, *Burkholderia*, *Pantoea*, and *Pseudomonas*). The top five strains with the strongest phosphate-solubilizing capability all belonged to *Burkholderia* (Figure 4). Strain CO1-5 (*Burkholderia* sp.) showed the highest phosphate-solubilizing capability with a PSI value of 3.05 ± 0.13 after 6 days incubation, higher than the report from Sengupta *et al.* [24] and Yasmin and Bano [25] that tested on Picovskaya's agar media using tricalcium phosphate as the sole P source. Previous studies reported that the most significant solubilizers of phosphate are mainly belonging to *Bacillus* and *Pseudomonas* [12, 26-28]. However, in our study, *Burkholderia* species were easier to be isolated and showed a high phosphate-solubilizing potential (Figure 4). *Burkholderia* strains formed no specific clade from the result of phylogenetic analysis (Figure 2), suggesting no specific lineage of *Burkholderia* PSB existed in the rhizosphere of *C. oleifera*. The average value of PSI of *Burkholderia* (61 strains) isolated from *C. oleifera* rhizospheres was 1.65, higher than those of *Bacillus* (17 strains, PSI: 1.26), *Paraburkholderia* (7 strains, PSI: 1.35), and *Pseudomonas* (14 strains, PSI: 1.25). The results indicated the selection on PSB by the rhizosphere

of *C. oleifera* may contribute to Pi absorption from soil.

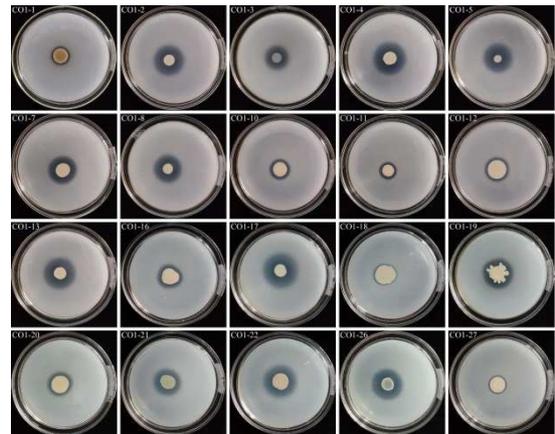


Figure 3. Phosphate-solubilizing halos formed on NBRIP agar media using the point inoculation method by PSB isolated from sample CO1.

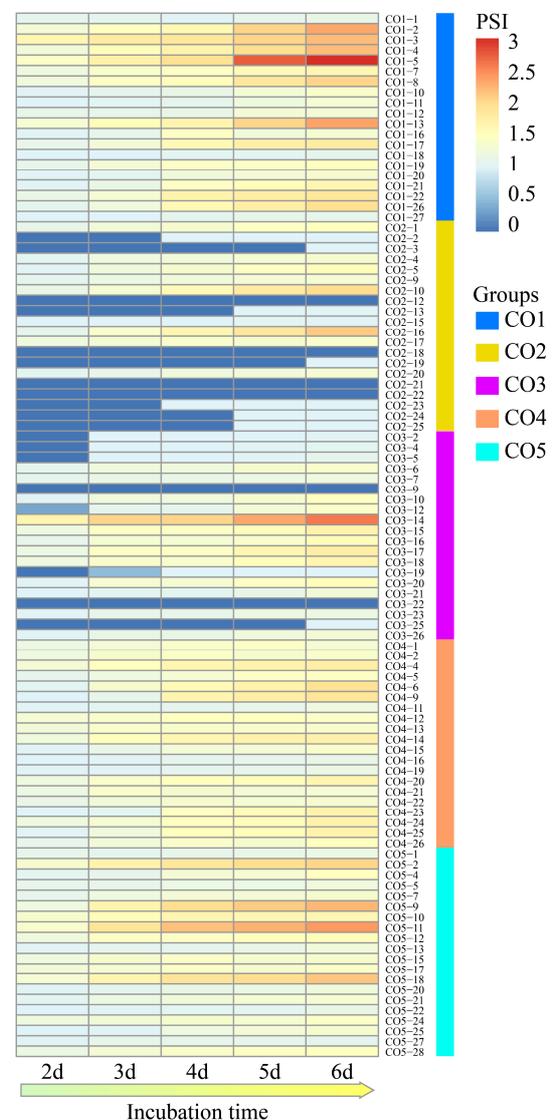


Figure 4. Heatmap of phosphate solubilization index (PSI) values of the 100 PSB isolated from rhizospheres of *C. oleifera*.

Although *Burkholderia* strains had a strong phosphate-solubilizing capability and were unevenly distributed in rhizospheres (Figure 4), there was no significant difference in the overall phosphate-solubilizing potential of PSB in different *C. oleifera* rhizospheres (Figure 5), suggesting that wild *C. oleifera* trees can adjust PSB composition to maintain fair phosphate solubilization. Despite *C. oleifera* contains Pi transporters that can efficiently absorb soluble Pi in the root-soil interface [29], the soluble Pi cannot meet the requirement of plant growth due to its low concentration in the soil. Plant roots can secrete plant-derived metabolites to affect the composition of rhizosphere bacteria and promote Pi acquisition [30]. The regulation of rhizosphere bacteria may be one of the important mechanisms for *C. oleifera* adapting to low P stress. *C. oleifera* usually forms mycorrhiza with arbuscular mycorrhizal fungi (AMF), the fungal symbionts can promote their nutrient absorption [31].

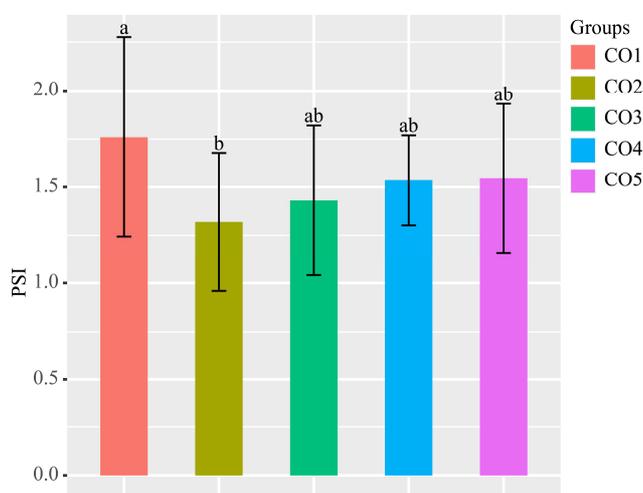


Figure 5. Difference of average phosphate-solubilizing ability of PSB from different rhizospheres.

AMF can promote host plant nutrition by hyphal uptake or regulating beneficial bacteria in the rhizosphere [32-34]. For example, Emmett et al. [35] found that AMF can selectively enrich members of the Proteobacteria. Studies based on culture-dependent methods found that PSB in the rhizosphere of *C. oleifera* were mainly *Burkholderia*, *Enterobacter*, and *Pseudomonas* in Proteobacteria [15, 17, 18]. In this study, similar results were obtained that strains isolated from the rhizosphere of *C. oleifera* were mainly *Burkholderia*, *Paraburkholderia*, *Pantoea*, and *Pseudomonas*, indicating that the rhizosphere of *C. oleifera* may enable to select members of Proteobacteria to facilitate the mobilization of insoluble Pi. The phosphate-solubilizing capability of PSB is unstable, easily weakened, or lost during rescreening or incubation [17, 36]. We also found a similar phenomenon that some strains lost their phosphate-solubilizing capability during incubation, all of which were *Bacillus*, indicating that the capability of *Bacillus* strains from the rhizosphere of *C. oleifera* was unstable.

5. Conclusions

This study showed a large population size of PSB existed in the rhizosphere of *C. oleifera*. A total of 100 strains of PSB isolated from rhizosphere soils belonged to *Bacillus*, *Burkholderia*, *Pantoea*, *Paraburkholderia*, and *Pseudomonas*, of which *Burkholderia* isolates were the predominant culturable group and shown higher phosphate-solubilizing capability. Of the isolated PSB, *Bacillus* species had the lower phosphate-solubilizing capability and were easy to lose the capability. Our result suggested that *Burkholderia* microbes were the efficient PSB resource that can be utilized for facilitating the uptake of P by *C. oleifera*. In future study, more work should be performed on selecting efficient phosphate-solubilizing isolates for pot experiments to verify their capacity and stability in promoting the absorption of P by *C. oleifera*.

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