Effects of Phosphate-Solubilizing Bacteria and N$_2$-fixing Bacteria on Nutrient Uptake, Plant Growth, and Bioactive Compound Accumulation in *Cyclocarya paliurus* (Batal.) Iljinskaja

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Abstract: Research Highlights: We firstly interpreted nutritional mechanisms involved in growth regulation and phytochemical accumulation in *Cyclocarya paliurus* (Batal.) Iljinskaja under three inoculant types, and selected bacterial inoculations for multiple purposes of *C. paliurus* plantation. Co-inoculation with phosphate-solubilizing bacteria (PSB) and N$_2$-fixing bacteria (NFB) performed better in growth promotion and nutrient uptake than single bacterial inoculation.

Background and Objectives: *C. paliurus* is a well-known medicinal plant as it accumulates bioactive compounds (BC) such as flavonoids, triterpenoids, and polysaccharides, in its leaves. However, the effects of plant growth-promoting rhizobacteria (PGPR) on the growth and BC yields in *C. paliurus* are not known. To fill this gap, the effects of different inoculants should be examined.

Materials and Methods: A pot experiment was conducted and two-year-old *C. paliurus* seedlings were inoculated with three inoculant types (PSB, NFB, PSB + NFB). After four rounds of inoculation, the growth characteristics and concentrations of flavonoids, triterpenoids, and polysaccharides, as well as the nutrients in soil and leaves, were measured. Results: The inoculations resulted in the elevation of soil available nutrients, with improvements in plant growth, BC yield, and N and P uptake in leaves. However, the changes in BC yields were mainly a result of elevated leaf biomass rather than BC concentrations, and leaf biomass was regulated by C:N:P stoichiometry. Co-inoculation with PSB and NFB was applicable for leaf production, while inocula related to NFB resulted in higher BC yields than PSB and control.

Conclusions: Our results implied that bacterial inoculants improved plant growth and BC yield by altering the nutrients in soil and leaves, while three inoculant types showed a different pattern in which co-inoculation with four strains presented a greater performance than single bacterial addition.

Keywords: *Cyclocarya paliurus*; phosphate-solubilizing bacteria; N$_2$-fixing bacteria; bioactive compounds

1. Introduction

*Cyclocarya paliurus* (Batal.) Iljinskaja, a deciduous tree, belongs to the family Juglandaceae and is mainly distributed across the subtropical mountainous areas of China [1]. Its leaves are often used in herbal tea [2] and as an essential ingredient of medicine to treat diabetes in China [3]. A growing body of evidence indicates that diverse bio-activities (including antidiabetic, antioxidant, and antimicrobial activities) were found in the extracts of *C. paliurus* leaves [4]. These extracts are mainly comprised of...
flavonoids, triterpenoids, and polysaccharides, which contribute to protecting humans against chronic diseases [3,5]. Based on these beneficial effects on human health, there is an increasing demand for the production of leaf and bioactive compounds (BC) in C. paliurus leaves for their medicinal applications.

However, the majority of C. paliurus plantations have to be assigned to poor sites in the mountainous areas in Southern China, as part of the Grain for Green Project (GTGP). These regions are perceived to be infertile due to low levels of organic C and available nutrients (mainly N and P) [6,7], which are deemed to be the essential nutrients for plant growth [8]. Chemical N and P fertilization are competent to promote plant growth and obtain optimal yield. Many types of studies have highlighted the positive effects of chemical fertilization on the yield and growth in medicinal plants. Deng et al. [9] reported that inorganic NPK fertilizer is conducive to optimizing the yields of targeted health-promoting substances in C. paliurus. Kumar et al. [10] demonstrated that the highest seed yield and seed weight of fenugreek (Trigonella foenumgraecum L.) were found with chemical NPK fertilization at the rate of 50:50:25 kg·ha⁻¹. However, after long-term chemical fertilization, soil degradation and pollution have been getting worse. At the same time, limited nutrients in the soil are sustainably exploitable for plant uptake due to N-leaching, ammonia volatilization, and P-immobilization [11,12]. Recently, owing to advances in the understanding of microorganism–plant interactions, researchers’ attention has been attracted by increasing applications of biological and natural fertilizers, because of their outstanding performance in crop growth and smaller ecological footprint compared with chemical fertilizers.

Of the recommended strategies, the utilization of bio-fertilizer based on plant growth-promoting rhizobacteria (PGPR) has proven to be an efficient and eco-friendly management practice [13]. These bio-fertilizers contain living beneficial microorganisms that can colonize the rhizosphere and stimulate crop growth by increasing the supply of available nutrients to the host plant when applied to the soil [13]. PGPR, such as N₂-fixing bacteria (NFB) and phosphate-solubilizing bacteria (PSB), have already been sufficiently studied. For instance, Azotobacter chroococcum and Azospirillum brasilence, two free-living aerobic NFB can be found in most soil and have the ability to convert inert N₂ into available forms for plants [14]. Bacillus megaterium and Pseudomonas fluorescens (PSB) are notable for the ability to solubilize unavailable phosphates in soil, as well as produce a wide variety of metabolites like auxin [15,16]. The application of PGPR as a bio-fertilizer on medicinal seedlings has resulted in a higher yield of BC and plant growth in different crops, such as Glycyrrhiza uralensis Fisch [17], Juglans regia L. [18], and T. foenumgraecum L. [16]. Some researchers have proven that mixed inoculation of PSB and NFB was an alternative bio-fertilizer for supplying N and P to walnut plants [18]. However, there is no information about the effects of bio-fertilizer, especially for co-inoculation with PSB and NFB, on plant growth and BC of C. paliurusx.

The BC in this study included total flavonoid, total triterpenoid, and water-soluble polysaccharide in C. paliurus leaves. Among flavonoids, seven flavonoid monomers of which were identified in the previous study [19] and presented important values for medicinal use [20,21], were thus chosen in this study. The aim of this study was to investigate the effects of PSB (B. megaterium and P. fluorescens), NFB (A. chroococcum and A. brasilence), and co-inoculation with PSB and NFB accompanied with organic fertilizer, on the growth characteristics, nutrients in soil and leaves, and the yield and concentration of BC in C. paliurus leaves. We hypothesized that (1) PGPR inoculated in the rhizosphere can facilitate plant growth and BC yield of C. paliurus, (2) such a promotion may directly or indirectly derive from altered internal C:N:P stoichiometry in leaves, and (3) co-inoculation with PSB and NFB will result in greater performance than when these strains were used alone. Our findings build the connection between PGPR and plant secondary metabolites and offer opportunities to choose a sustainable way to reform the soil and establish C. paliurus plantation for pharmaceutical supply.
2. Materials and Methods

2.1. Seedlings, Growth Media, and Microorganism’s Preparation

On November 1, 2017, two-year-old *C. Paliurus* seedlings were chosen from Muchuan, Sichuan, China (28°96’ N, 103°98’ E), based on the previous research [22]. The initial heights of the seedlings ranged from 32–38.5 cm and the ground caliper ranged from 5.02–6.1 mm.

The medium for plant growth in pot-experiment was a mixture of soil, sand, organic fertilizer, and coconut residuum (7:2:0.8:0.2, v/v/v). The soil was collected from the plow layer of soil (0–20 cm) at *C. Paliurus* plantation in Nanjing, China (31°35’ N, 119°10’ E), more information was presented in our previous study [23]. The organic fertilizer added to the medium was used to improve the survival and multiplication of bacteria. One seedling was planted in each pot (top diameter: 25 cm, bottom diameter: 20 cm, height: 30 cm) containing 5 kg of growth medium. The basic physicochemical properties of medium were as follows: pH 5.98, total C of 18.9 g kg⁻¹, total N of 0.79 g kg⁻¹, total P of 0.30 g kg⁻¹, total of K 0.10 g kg⁻¹, available N of 12.68 mg kg⁻¹, and available P of 5.56 mg kg⁻¹.

The bacterial strains used in this study were *Bacillus megaterium* W17 [18], *Pseudomonas fluorescens* W12 [24], and *Azotobacter chroococcum* HKN-5 [25] and *Azospirillum brasilence* CW903 [26]. These bacteria have been documented with the ability of improving soil nutrients such as N and P, and none of these bacterial strains showed any antagonistic effects against one another [23]. Prior to use, bacteria strains were incubated in lysogeny-broth medium (LB, pH 7.0, comprised of 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) to the mid-exponential growth phase. At the same time, the bacterial population was examined in a lab using the plate count serial dilution method [27] while experimenting on building a standard curve between optical density and bacterial quantities. After that, the inoculants were diluted by sterile LB medium to a final concentration of 1×10⁸ colony forming units (CFU)·mL⁻¹ according to the standard curve.

2.2. Site Description and Experimental Design

The seedling nursery was located in Lishui, Nanjing, China (31°35’ N, 119°10’ E), where the *C. paliurus* plantation was established. This area is a typical transition zone from the north subtropics to the subtropics where the climate is mild and humid, with abundant rainfall (1037 mm/year) and sunshine (2146 h/year), and the annual average temperature being approximately 15.4 °C.

The experiment was laid out in a three-block pattern based on a randomized complete block design. Seven treatments included three inoculant types (PSB, NFB, PSB + NFB) and two control (without bacteria but LB medium and water), each treatment containing 60 seedlings that were equally divided into three blocks. Details are shown in Table 1. After seedlings were well established, bio-fertilization with seven treatments were conducted four times with an interval of about 45 days (April 4, May 19, July 6, and August 19, 2018, respectively). Specifically, 50 mL (1×10⁸ CFU·mL⁻¹) inoculations in total were circularly injected into rhizosphere in each pot according to bio-fertilization regimes in Table 1.

Table 1. Fertilizing doses of seven bio-fertilization regimes (mL-pot⁻¹).

| Inoculant Type | Treatment | M: *Bacillus megaterium* | F: *Pseudomonas fluorescens* | C: *Azotobacter chroococcum* | B: *Azospirillum brasilence* | LB | Water |
|---------------|-----------|--------------------------|-----------------------------|-----------------------------|-----------------------------|----|-------|
| PSB ¹ | M         | 50                       | 0                           | 0                           | 0                           | 0  | 0     |
|        | MF        | 25                       | 25                          | 0                           | 0                           | 0  | 0     |
| NFB ² | C         | 0                        | 0                           | 0                           | 50                          | 0  | 0     |
|        | CB        | 0                        | 0                           | 0                           | 25                          | 25 | 0     |
| PSB + NFB ³ | MFCB      | 12.5                     | 12.5                        | 12.5                        | 12.5                        | 0  | 0     |
| Control ⁴ | LB        | 0                        | 0                           | 0                           | 0                           | 50 | 0     |
|        | CK        | 0                        | 0                           | 0                           | 0                           | 0  | 50    |

¹ Phosphate-solubilizing bacteria (PSB): inoculated with strain *B. megaterium* (M), *B. megaterium*, and *P. fluorescens* (MF).
² N₂-fixing bacteria (NFB): inoculated with strain *A. chroococcum* (C), *A. chroococcum*, and *A. brasilence* (CB).
³ PSB + NFB: co-inoculation with four strains (MFCB).
⁴ Control: inoculated with LB medium and water.
According to a previous study [23], the bacterial population hit a peak at days 30–45 of incubation. Available soil N and P contents and related enzyme activity were significantly increased in co-inoculations with PSB and NFB. Hence, the bio-fertilization frequency (every 45 days) and inoculants types (Table 1) were determined in this pot-experiment based on previous results.

2.3. Measurement of Soil Available Nitrogen and Phosphorus

For the measure of soil available N (SAN) and soil available P (SAP) in the rhizosphere, five soil samples (5–10 cm) were collected randomly for each treatment on September 8, 2018 and kept at 4 °C prior to analysis. SAN (NH$_4^+$ + NO$_3^-$) was determined by extraction with 2M KCl in 1:5 (w/v) soil-to-solution ratio, shaking for 1 h at 200 rpm, and followed by quantification using a continuous flow analyzer (Bran + Luebbe AA3, Germany). SAP was extracted by ammonium fluoride and hydrochloric acid in 1:10 (w/v) and determined using the molybdenum-blue method [28].

2.4. Plant Growth and Leaf Harvest

Seedling height and caliper were measured for all healthy seedlings (about 27 seedlings for each treatment) at every fertilization time, and the total increment of growth was calculated by the difference of initial and final height/caliper. For biomass measurement, three seedlings of each treatment were excavated entirely on September 6, 2018, washed, and separated into four components (leaf, stem, thick root, and fine root). Afterward, all components were dried at 60 °C and weighed, respectively. The total dry mass of each seedling was calculated as the sum of leaf, stem, and root dry weight. The ratio of underground biomass to above-ground biomass (root/shoot ratio) was calculated.

After biomass assessment, all the leaves of *C. Paliurus* (three samples of each treatment) were ground and stored at room temperature for the following measurement of nutrients and bioactive compounds in leaves.

2.5. Measurement of Total Carbon, Nitrogen, and Phosphorus in Leaves

For the measurement of total carbon (C) and nitrogen (N) contents, each sample (50.0 mg) of leaves was wrapped up with a tin can and total C and N were determined by the elemental analyzer (vario MAX CN, Elementar, Hanau, Germany). For the measurement of total phosphorus (P) contents, each sample (1 g) was digested by HNO$_3$ and HClO$_4$ (5:1 in volume), and total P was determined by the molybdenum-blue method.

2.6. Extraction and Determination of Bioactive Compounds

Flavonoids were extracted from *C. paliurus* leaves using an ultrasonic-assisted method with 75% ethanol after removing fat-soluble impurities with petroleum ether. The total flavonoid concentration was determined using a colorimetric method with detection at 415 nm [29] and was calculated using the standard Rutin curve and expressed as a milligrams Rutin equivalent per gram of dry mass (mg/g). Seven flavonoid monomers (Figure 1), including quercetin (quercetin-3-O-glucuronide; quercetin-3-O-galactoside; quercetin-3-O-rhamnoside), kaempferol (kaempferol-3-O-glucuronide; kaempferol-3-O-rhamnoside; kaempferol-3-O-glucoside; kaempferol-3-O-rhamnoside), and isoquercitrin, were determined and identified by high-performance liquid chromatography system (HPLC, Waters, Milford, MA, USA) coupled with quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS) [19].

The extraction of water-soluble polysaccharide in *C. paliurus* leaves was carried out as described previously by Fu et al. [30] and the polysaccharide concentration was determined by the phenol–sulfuric acid method. For triterpenoid extraction, 2.0 g of leaves were extracted using an ultrasonic-assisted method. Briefly, 50 mL of 75% ethanol was added to each sample, and the extraction was conducted for 45 min at 65 °C and repeated twice. The total triterpenoid concentration was determined according to a previously described laboratory procedure using a colorimetric method with slight modifications [31].

The yields of these bioactive components in leaves were calculated as the concentration multiplied by the biomass of leaves.
2.7. Statistical Analysis

The Shapiro-Wilk test and Levene’s test were used to test the normal distribution of data and homogeneity of variances, respectively. When there were significant effects \((p < 0.05)\), Duncan’s multiple range test was applied to determine the differences among individual treatment means. Tamhane’s \(T_2\) was used to test for differences among treatments when variances of tested data were not equal. All statistical analyses were considered significant at \(p < 0.05\). The pairwise correlations of plant growth characteristics, nutrient uptake, concentrations, and yields of bioactive components were elucidated using Spearman’s correlation analysis. All statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Soil Available N and P

The contents of soil available N (SAN) and soil available P (SAP) in the rhizosphere are presented in Figure 2. After four rounds of bio-fertilization, SAN and SAP were significantly increased compared to the control; however, different patterns were noted. Dual inoculation with two NFB (treatment CB) resulted in the highest contents of SAN and showed an obvious advantage over other inoculants. On the other hand, the highest content of SAP was observed in co-inoculation with PSB and NFB (treatment MFCB), while single inoculation (treatment M and C) caused lower effects.
Figure 2. Contents of (a) soil available N, and (b) soil available P in the rhizosphere as affected by different inoculant types (PSB, NFB, PSB + NFB). Different lowercase letters denote significances of soil available N and soil available P among treatments at $p < 0.05$ level.

3.2. Plant Growth and Biomass

The bio-fertilization had a significant effect on plant growth (Figure 3A,B) and biomass production (Figure 3C). The total increment of plant height ranged from 71.81 cm in CK to 93.67 cm in MF, and the level of treatment MF, C, and MFCB were significantly higher than the control (LB and CK) ($p < 0.05$). Significant increments of seedling caliper were noted for all bio-fertilizer treatments, ranging from 7.05 mm in CK to 8.31 mm in MF. For both height and caliper, the highest increments all occurred in MF, reaching 93.67 cm and 8.31 mm, respectively.

Figure 3. Plant growth, biomass and partitioning of C. paliurus as influenced by inoculants. (A): The total increment of plant height. (B): The total increment of the caliper. (C): Comparison of different biomass components (leaf, stem, fine root, and thick root) and above/under/total biomass among all treatments; different lowercase letters inside the bar denote significant variances of biomass component among treatments at $p < 0.05$; different capital letters above/under the bar denote significant differences of above/under-ground biomass between treatments at $p < 0.05$; the comparison of total biomass presented at the bottom. (D) Root/shoot ratio was calculated as the ratio of underground biomass to ground biomass. Different lowercase letters above the bar in A, B, and D denote significant differences among treatments at $p < 0.05$. 
Compared with CK and LB, co-inoculation with PSB and NFB (treatment MFCB) resulted in the highest total biomass accumulation (74.9 g per plant) and higher ground biomass distribution (47.83 g), while the application of NFB (C and CB) significantly increased the underground biomass. However, no positive effect of PSB application (M and MF) on plant biomass was found during the investigation (Figure 3C). Consequently, the root/shoot ratio was significantly increased in CB and decreased in MFCB, respectively (Figure 3D). Noteworthily, the MFCB treatment significantly increased the leaf biomass accumulation, which is the target production for food and medicinal use.

### 3.3. C:N:P Stoichiometry in *C. paliurus* Leaves

The contents of C, N, and P in *C. paliurus* leaves for each treatment were measured, and the C/N, C/P, and N/P ratios were calculated (Table 2). According to the results, the N and P contents in leaves were increased in three inoculant types (PSB, NFB, PSB + NFB) compared to the control (*p* < 0.05), ranging from 21.00–27.81 g·kg\(^{-1}\) (N) and from 1.57–1.95 g·kg\(^{-1}\) (P), respectively. Co-inoculation of PSB and NFB resulted in higher N and P contents in leaves than single bacterial addition (M and C, *p* < 0.05). However, the dual inoculation of two PSB (MF) or two NFB (CB) possessed no significant advantage over single bacteria.

Table 2. Contents of total carbon, nitrogen, phosphorus, and their ratios in *C. paliurus* leaves.

| Inoculant Type | Treatment | Carbon (g·kg\(^{-1}\)) | Nitrogen (g·kg\(^{-1}\)) | Phosphorus (g·kg\(^{-1}\)) | C/N | C/P | N/P |
|---------------|-----------|------------------------|--------------------------|-----------------------------|------|-----|-----|
| PSB           | M         | 464.28a                | 23.04d                   | 1.86b                       | 20.18b | 250.14cd | 12.41d |
|               | MF        | 455.90a                | 23.82cd                  | 1.76c                       | 19.16bc | 258.50bc | 13.5ab |
| NFB           | C         | 464.19a                | 25.83b                   | 1.91ab                      | 17.97c | 243.42de | 13.54ab |
|               | CB        | 459.50a                | 25.08bc                  | 1.73c                       | 18.33c | 265.69b  | 14.50a |
| PSB + NFB     | MFCB      | 464.23a                | 27.81a                   | 1.95a                       | 16.70d | 237.63e  | 14.23ab |
| Control       | LB        | 465.78a                | 21.44e                   | 1.62d                       | 21.73a | 287.04a  | 13.23cd |
|               | CK        | 462.08a                | 21.00e                   | 1.57d                       | 22.00a | 295.26a  | 13.42cd |

1 Different lowercase letters in the same column denote significant differences among treatments at *p* < 0.05 level.

On the other hand, the applications of three inoculant types caused a slight but nonsignificant increment of C contents in leaves (ranging from 455.9–465.8 g·kg\(^{-1}\)). As a result, the C/N ratios and C/P ratios of controls (LB and CK) were significantly higher than all treatments with inoculations, while N/P ratios indicated a contrary pattern.

### 3.4. Flavonoids

Concentrations and yields of seven flavonoid monomers and total flavonoid in *C. paliurus* leaves are presented in Figure 4. Total flavonoid concentrations were slightly elevated (*p* > 0.05) after bio-fertilization and ranged from 19.0 mg·g\(^{-1}\) in M to 23.23 mg·g\(^{-1}\) in CB, while a significant increment of total flavonoid yield was observed in all treatments except M (Figure 4h). Furthermore, inocula related to NFB (C, CB, and MFCB) resulted in higher yields than PSB and the control.

In terms of the seven flavonoid monomers, significant variances of concentrations and yields were detected among all treatments (Figure 4a–g). However, the accumulation of flavonoid monomers showed different variation patterns between PSB, NFB, and PSB + NFB. The co-inoculation of PSB and NFB (MFCB) improved the accumulation of monomers in both concentration and yield, while PSB had negative effects. On the other hand, inoculation with NFB possessed a significant advantage over inoculation with PSB. The highest concentration and yield of all flavonoid monomers were observed in kaempferol-3-O-glucuronide in MFCB, which obtained 2.0 mg·g\(^{-1}\) and 21.6 mg·plant\(^{-1}\), respectively (Figure 4d).
3.5. Water-Soluble Polysaccharide and Triterpenoid

The effects of bio-fertilization on water-soluble polysaccharide and triterpenoid concentrations in *C. paliurus* leaves were not significant (Figure 5, *p* > 0.05). However, inocula related to NFB resulted in higher yields of polysaccharide and triterpenoid than PSB and the control. The highest yield of total triterpenoid and polysaccharide in *C. paliurus* leaves were achieved in treatment with C, followed by MFCB, whereas the lowest yield was noted in CK. Compared with CK, total triterpenoid yields in treatment C and MFCB increased by 81.6% and 63.6%, while the polysaccharide yields increased by 103.9% and 84.7%, respectively.
Figure 5. Concentrations (mg·g⁻¹) and yields (mg·plant⁻¹) of total triterpenoid (A) and water-soluble polysaccharide (B). Different lowercase/capital letters denote significant differences of concentrations/yields among treatments at \( p < 0.05 \).

4. Discussion

To increase the medicinal values of *C. paliurus*, optimizing production of the plantation is a research focal point, especially in cultivation management strategies such as chemical fertilization, light quality, and artificial shade [9,32,33]. As a sustainable method for amending the soil, PGPR were applied extensively in crop growing and have been proven to have positive effects on crop output as well as soil properties [12,17,34]. In this study, we focused on not only biomass improvement (leaf) but also the accumulation of BC in *C. paliurus* leaves by the addition of PGPR to the growing medium.

4.1. PGPR Symbiosis Increased Nutrients in Soil Which Improved Plant Growth

As a multifunctional medicinal plant, *C. paliurus* leaf is the principal organ for accumulating bioactive compounds (BC) [35]. Moreover, as the organ of photosynthesis, nutrients in leaves directly or indirectly affect C assimilation, phytochemical accumulation, and plant growth. Of all nutrients, N and P are indispensable in regulating plant growth and ecosystem productivity [36,37]. However, available nutrients are often limited under poor soil conditions, which could be amended by bio-fertilizers.

Usually, the promotions in growth and biomass are supposed to derive from improved soil available nutrients after bacterial inoculation, so as to promote N and P uptake in the plant [38]. This is supported by our results that SAN, SAP, and N and P contents in leaves were increased under three inoculant types (Figure 2; Table 2). According to the correlation analysis, N and P contents in leaves were significantly correlated with SAN, SAP, and growth characteristics (Table S2, \( p < 0.05 \)). Therefore, the improvement of plant growth mainly resulted from elevated N and P uptake, which were induced by increased nutrients in the soil. Similar results were reported in different plants, whose growth characteristics and biomass correlated with the internal nutrients uptake altered by PGPR [39,40].

For the response of improvement in growth to PSB and NFB inoculation, the widely accepted speculation is that plant growth and biomass accumulation would be affected by C:N:P stoichiometry, induced by fertilization, photosynthesis, and microorganisms [41,42]. As shown in Table 2, Table S2, and Figure S1c,d, the leaf biomass was positively correlated with both N and P contents in leaves (\( p < 0.05 \)), and negatively correlated with C/N and C/P ratio (\( p < 0.05 \)), while no significant correlations were observed between leaf biomass and N/P ratio. Clearly, PGPR are responsible for facilitating N and P availability, trigging N and P uptake by the host plant, then regulating plant growth and biomass accumulation by altering the internal nutrient balance [43].

4.2. PGPR Additions Improved the BC Output Mainly by Increasing the Leaf Biomass rather than Concentrations

Main BC in *C. paliurus* leaves, such as flavonoids, triterpenoids, and polysaccharides are responsible for numerous medicinal effects. Much literature has concluded that the accumulation of these BC in *C. paliurus* leaves was influenced by genetic, cultivation practices and climatic factors [9,22,30,33].
Among these, fertilizations play vital roles in the oriented cultivation of C. paliurus plantation for medicinal use.

It is known that plant secondary metabolites could be induced by adverse environmental conditions and regulated by internal nutrients balance [41]. Previous studies indicated that C, N, S, and P contents in plants were related to both primary growth and secondary metabolites [44,45]. Xie et al. reported that the improved root P status to arbuscular mycorrhizal fungi could affect plant C balance and induce more C partitioning to secondary metabolism [46]. Plants accompanied by soil microorganisms could be assisted with nutrient acquisition, while N and P uptake could affect the allocation of C resources and cause changes in C:N:P stoichiometry [7]. These changes were considered as the nutritional benefits of PSB and NFB symbiosis to host plants, and affected primary growth as well as secondary growth [13,39].

As presented in this study, the yield of total flavonoid, polysaccharide, and triterpenoid was significantly elevated under inocula related to NFB (C, CB, MFCB), while there was little influence on their concentrations (Figures 4h and 5). This is in accord with the results of regression analysis, in which N and P contents in C. Paliurus leaves were positively correlated with leaf biomass and yields of BC, but there were no significant correlations with concentrations (Figure S1). Bio-fertilization is in favor of plant primary growth but not the accumulation of BC. Thereby, the increments of the yield of BC mainly resulted from the promotion of leaf biomass rather than their concentrations.

In contrast, for the seven flavonoid monomers, significant variances in both concentrations and yields were detected among all treatments (Figure 4a–g). However, we found only the concentration of isoquercetin was significantly correlated with N uptake, while other monomers indicated no significance (Table S1). Hence, different PGPR, such as PSB and NFB, may indirectly influence the biosynthesis of flavonoids through manipulating other factors, such as gene expression [41], enzyme activity [35,47], or phytohormone [48]. For all flavonoid monomers, they possess the common biosynthetic pathway with little difference. Flavonoids are usually conjugated with glucose and biosynthesized from phenylalanine and malonyl-CoA produced by the shikimate pathway in plants [49]. Increased nutrients uptake in plants could contribute to the production of the precursor, such as phenylalanine, which is the common precursor of primary metabolism and secondary metabolism [50].

Plant growth and biomass accumulation mainly depend on primary metabolism, while plant defense and adaptation rely on secondary metabolism [51]. Many theories have been proposed to explain potential trade-offs between plant primary growth and secondary metabolite synthesis [52]. It worth noting that economic returns may not increase with a higher concentration of secondary metabolites in plants, as a higher concentration is often offset by lower biomass under stress conditions [53]. Thus, to achieve a high yield of objective ingredients, cultivation practices in soil/media is required, but the relationship between leaf production and phytochemical concentration in leaves should be balanced when the plantation is used for medicinal production.

### 4.3. Selections of PGPR Could be Considered for Multiple Purposes of C. paliurus Plantation

The effects of bio-fertilization depend on plants, soil types, and harvest targets [34]. As a multi-functional woody plant, C. paliurus could be utilized for timber, tea, as well as medicine [30]. Although inoculations resulted in increments of plant growth, the effects of PSB and NFB differed on growth regulations and accumulations of BC. For timber use, a fertilization strategy in favor of vegetative growth, reflected in tree height, diameter, and volume of timber, should be considered as a priority. As shown in our work, MFCB and MF treatments improved growth and above-ground biomass accumulation of C. paliurus under yellowish-brown clay soil mixed with organic fertilizer (Figure 3). Hence, treatment MF and MFCB are alternatives in plantation for timber use.

Different from plant growth in most crops, more attention should be paid to harvesting a high yield of BC for medicinal plants, such as C. paliurus. However, fewer effects of fertilization on concentrations of medicinal components were reported [16,35], as revealed in our study. Similarly, biomass improvement of the main organ for the collection of medicinal components by fertilizers could
achieve a high yield of target components. Moreover, our results (Figure 3) and predictions (Table S3) proved the feasibility of fertilizers.

In addition, the selection of PGPR should be considered according to the soil conditions and harvest targets. As found in C. paliurus, inocula related to NFB (C, CB, MFCB) resulted in higher BC yields than PSB and the control, while the highest production of leaves was in MFCB, twice as much as the control (Table S3).

No matter what C. paliurus plantation is focused on, soil conditions should be taken into account. The present study found that co-inoculation with PSB and NFB resulted in higher SAP than the others, while treatment CB achieved the highest value of SAN. Based on our previous study, synergistic effects between PSB and NFB may contribute to higher availability of soil nutrients and stimulate plant growth [23]. Several studies reported that inoculating plants with both PSB and NFB could result in higher available N and P contents in soil and nutrient uptake in plants [18,54]. This is because mixed microbial cultures allowed their components to interact with each other synergistically via physical or biochemical activities, thereby simultaneously improving viability in soil [55].

5. Conclusions

In this study, PGPR inoculations resulted in a significant increment of soil nutrients, with an improvement in plant growth, biomass, and N and P uptake in C. paliurus leaves. Co-inoculation with PSB and NFB presented better performances than single-bacterial addition. Significant influences of PGPR on the concentrations of flavonoid monomers were noted, while no effects were found in the concentrations of bioactive compounds. The changes in bioactive compound yields were mainly a result of leaf biomass promotion rather than their concentrations, and leaf biomass was regulated by C:N:P stoichiometry in leaves. Co-inoculation with PSB and NFB was more appropriate for leaf production, while inocula related to NFB resulted in higher bioactive compound yields than PSB and the control. This study firstly interpreted nutritional mechanisms involved in growth regulation and phytochemical accumulation of C. paliurus under bio-fertilization and provided selections of PGPR for multiple purposes of C. paliurus plantation. Future research should focus on non-nutritional mechanisms involved in PGPR symbiosis affecting secondary metabolite accumulation.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/9/772/s1, Figure S1: The relationships between N, P contents in leaves vs total yields/concentrations of bioactive compounds, and C:N:P vs leaf biomass, Table S1: Spearman’s correlations (r value) between C, N, P uptake and concentrations/yields of bioactive compounds, Table S2: Spearman’s correlations (r value) between C, N, P uptake, soil available nutrients, and plant growth characteristics, Table S3: Predication of bioactive compounds yield and leaf production of 2-year-old C. paliurus under same bio-fertilizer treatments.

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