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# Effect of Cork Flour Supplementation on the Growth Indices and Rhizosphere Bacterial Communities of *Quercus variabilis* Seedlings

**Yuying Che [1], Yong Kong [1], Shangwen Song [2], Xianfeng Yi [1], Jing Zhou [1,*] and Baoxuan Liu [3,*]**

[1] School of Life Sciences, Qufu Normal University, Jining 273165, China
[2] Jining Forestry Protection and Development Service Center, Jining 272000, China
[3] Shandong Cork Oak Industrial Technology Research Institute Co., Ltd., Jining 272075, China
* Correspondence: jingzhou-2004@163.com (J.Z.); laucork@pku.org.cn (B.L.)

**Abstract:** Substrate and rhizosphere microorganisms are key factors affecting seedling growth; however, the effects of seedling substrates and rhizosphere bacteria on the growth of *Quercus variabilis* are not completely understood. Here, *Q. variabilis* seedlings were grown in substrates with and without cork flour, as follows: H substrate (charcoal soil/cork flour/perlite, 1:1:2), S substrate (cork flour/perlite, 1:1), and the control (CK) substrate (charcoal soil/perlite, 3:2). High-throughput sequencing and qPCR were used to investigate the effects of these substrates on seedling growth, physiological indices, and rhizosphere bacterial communities. Root and shoot weights of seedlings grown in H and S substrates were significantly higher than those of seedlings grown in CK. Moreover, H was conducive to chlorophyll synthesis in seedling leaves, and the transpiration rate and intercellular $CO_2$ concentration of the leaves of seedlings grown in H were higher than those of seedlings grown in CK. The number of rhizosphere bacterial 16S rRNA copies was significantly greater in the case of seedlings grown in S than for those grown in H and CK. As well, rhizosphere bacterial richness was higher in seedlings grown in H and S than in those grown in CK. Thus, cork-flour-supplemented substrates are beneficial for seedling growth and development, seedling rhizosphere bacterial abundance and diversity, and the abundance of nitrogen and phosphorus metabolism-promoting microbial taxa.

**Keywords:** *Quercus variabilis*; seedling substrate; rhizosphere microorganisms; artificial breeding; bacterial diversity



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## 1. Introduction

With the rapid development of the global economy, greenhouse gas emissions have increased, posing a severe threat to human survival and development. To build a sustainable society, the government of China has committed to the goal of reducing carbon emissions and achieving carbon neutrality. *Quercus variabilis*, a deciduous tree of the Fagaceae family, is a highly valued (A-class) species with multiple uses in China; the roots, branches, leaves, trunk, bark, seeds, and seed shell have important ecological functions, such as water and soil conservation and ecological restoration [1]. Further, it is an important species for carbon neutrality, as its $CO_2$ absorption is five times the normal value after the bark is peeled. Thus, *Q. variabilis* plays a vital role in both ecological conservation and industrial production; however, there are multiple issues related to the arboriculture of *Q. variabilis*, such as the degradation of natural secondary forests of this species [2], including the decline in stand productivity, as well as the lack of appropriate scientific management. Furthermore, given the challenges involved in the breeding of high-quality and resilient *Q. variabilis* seedlings, selective seedling breeding and the development of new varieties are urgently required. In particular, strengthening seed conservation and expanding the breeding and industrial management of *Q. variabilis* are of paramount importance. At present, research on *Q. variabilis* is focused on physiological, biochemical [3,4], and ecological aspects [5].

In addition, *Q. variabilis* seed conservation and seedling breeding have been explored. Seedling substrates provide nutrients and water required for the growth and development of seedlings and produce a stabilising effect on the plant [6–8]. Lightweight substrates can improve the quality of seedlings, are easy to transport, and are pollution-free. Thus, screening for high-quality seedling substrates has emerged as a hot topic in container seedling research [9].

In current seedling cultivation in China, a number of substrates are used, such as vermiculite, peat, and perlite. A number of studies have been conducted on the effect of these substrates on different species, including *Q. cocciferoides* [10], *Sapium sebiferum* [10], *Ficus concinna* var. subsessilis [11], *Ulmus pumila* 'Jinye' [12], *Thuja sutchuenensis* [13], and *Acacia podalyriifolia* [14], yielding satisfactory results. However, the effects of substrates on plant growth are often influenced by various factors, such as the tree species subjected to breeding as well as the seedling breeding area and facilities [9]. Grass charcoal ash and perlite have long been considered excellent seedling substrates [7]. Recent studies on *Q. variabilis* seedling substrates have mostly used grass charcoal, vermiculite, perlite, coconut bran, charred rice husk, and other substrates. In the present study, we added cork flour, a by-product of *Q. variabilis* cork processing, to the seedling substrate. To the best of our knowledge, this is the first study in which mixtures of cork flour with charcoal soil and perlite (at specific ratios) have been compared with respect to the effect on the growth of *Q. variabilis* seedlings.

Rhizosphere microorganisms are essential for plant growth, as they promote plant growth and development and improve plant resistance [15]. However, there are relatively few studies on the differences among the rhizosphere microbial communities of seedlings grown on various substrates [16,17]. To this end, we used cork flour, charcoal soil, and perlite in different volume ratios to prepare three different substrates for growing *Q. variabilis*. Specifically, we investigated the effects of cork flour supplementation on the growth and physiological indices of seedlings as well as on the structure of the corresponding rhizosphere bacterial communities. The findings of this study serve as a technical reference in formulating high-quality *Q. variabilis* seedling substrates and breeding quality seedlings.

## 2. Materials and Methods

### 2.1. Experimental Materials and Treatments

*Quercus variabilis* seeds were provided by the Shandong Cork Oak Industrial Technology Research Institute Co., Ltd. (Jining, China). Full and pest-free seeds were selected. We had pre-determined several substrates that had been shown to result in a high survival rate (data were not published): H (charcoal soil/cork flour/perlite, 1:1:2 volume ratio), S (cork flour/perlite, 1:1), and CK (charcoal soil/perlite, 3:2). The prepared seedling substrates were placed into non-woven seedling containers (diameter, 6 cm; height, 10 cm). The containers were placed in a plastic tray (5 × 10 grids; length × width × depth: 54 × 28 × 8 cm). The seeds were soaked with carbendazim solution for 0.5 h, rinsed three times with clean water, and then placed in sand to promote germination, at which point the seedlings were transferred to the substrate treatment containers. The treatment containers were watered every 3 days, with the same watering volume and light conditions for each of the three treatments. After 4 months of growth, each treated seedling was randomly selected for the measurement of the below-ground and above-ground growth indices, and samples of rhizosphere were collected from soil adhering to the root crown, where rooting was sufficiently dense that all soil could be considered to be under the influence of roots. A part of these samples was stored in a freezer at −80 °C to determine the rhizosphere bacterial community structure, and the rest was naturally dried and used to test the conventional nutrient content.

### 2.2. Determination of the Nutrient Content of Q. variabilis Seedling Substrates

To determine the total nitrogen (TN) content, 1.0 g of the substrate was weighed, potassium permanganate–sulphuric acid was added, and the samples were analysed using

a semi-automatic Kjeldahl Azotometer [18]. The alkali-hydrolysable nitrogen (AN) content was determined using diffusion plate 0.01 mol·$L^{-1}$ HCL titration [19]. To determine the total phosphorus (TP) content, 1.0 g of the substrate was weighed, and perchloric acid–sulphuric acid digestion and Mo-Sb colorimetry were performed [20]. To determine the available phosphorus (AP) content, 5.0 g of the substrate was weighed, and ammonium fluoride–hydrochloric acid leaching and Mo-Sb colorimetry were performed [21]. To determine the total potassium (TK) content, 0.1000 g of the substrate was weighed into a platinum crucible, and nitric acid–perchloric acid-hydrofluoric acid digestion and flame spectrophotometry were performed [20]. To determine the available potassium (AK) content, 5.0 g of the substrate was weighed, and ammonium acetate (1.0 mol·$L^{-1}$) leaching and flame spectrophotometry were performed [19]. To determine the organic matter (OM) content, 0.5 g of the substrate was weighed, and potassium dichromate–sulphuric acid oxidation was performed [19]. All measurements were performed in triplicate.

### 2.3. Measurement of the Physiological Indices of Q. variabilis Seedling Growth

Seedling height and ground diameter were measured using a straightedge and vernier calliper, respectively. The crown was slightly gathered to measure the height from the joint of the rhizome to the tip of the highest branch of the seedling [22]. Root morphology was determined using the Epson Perfection V700 photo scanner in combination with the WinRHIZO Pro 2007 root analysis system [23]. The above-ground and below-ground parts of the seedlings were cut to determine the fresh weight, placed into sealed envelopes, dried at 105 °C for 15 min, dried to a constant weight at 70 °C, and analysed for moisture content [24]. The seedlings were placed in the dark for 30 min, and the light intensity was set at 1000 μmol·$m^{-2}$·$s^{-1}$. Seedling leaves were selected, and leaf chlorophyll fluorescence was measured using the Handy Plant Efficiency Analyser (Hansatech Instrument Ltd., Norfolk, UK) [25]. Three leaves were sampled via hole punch. The diameter of the hole punch was recorded, and the chlorophyll content of the leaves was determined using a mixture of acetone and dimethyl sulfoxide [26]. Between 9:00 and 11:00 a.m., the following photosynthetic parameters of the seedlings were determined using the TARGAS-1 portable photosynthetic assay system (PP System Corporation, Amesbury, MA, USA): net photosynthetic rate, transpiration rate, stomatal conductance, and intercellular $CO_2$ concentration [27]. The measurement conditions were as follows: temperature = 15–17 °C, atmospheric $CO_2$ concentration = 477 μmol·$mol^{-1}$, and light intensity = 1000 μmol·$m^{-2}$·$s^{-1}$. Briefly, 1.0 g of seedling leaves were weighed. Quartz sand and PBS were added to grind the samples, and the ground samples were centrifuged. To the supernatant, Coomassie brilliant blue reagent was added to determine foliar protein content [28]. Root morphology was repeated five times and other measurements were repeated 10 times.

### 2.4. Diversity of Rhizosphere Bacterial Communities of Q. variabilis Seedlings

DNA was extracted from 0.5 g of the substrate using the Fast DNA SPIN kit (MP, Santa Ana, CA, USA), and 16S rRNA genes were amplified using the primers 515FmodF (5′-GTG YCA GCM GCC GCG GTA A-3′) [29] and 806RmodR (5′-GGA CTA CNV GGG TWT CTA AT-3′) [30]. qPCR was used to detect the 16S rRNA copies of substrate bacteria to analyse the differences in bacterial populations amongst the three substrates [31]. The amplified PCR products were sequenced on the Illumina MiSeq PE 300 platform. The sequences obtained were submitted to the NCBI SRA database under the accession number PRJNA808877. The sequencing raw data were processed as follows: Species with fewer than five sequences in each group of three samples (operational taxonomic units, OTUs) or species with the sum of sequences in all samples being below 20 were removed to obtain the OTU table. Based on this table, alpha and beta diversity indices were measured to determine bacterial community diversity. Based on Euclidean distance, principal component analysis was performed with variance decomposition. Bacterial community functional analysis was performed by comparison against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to obtain KEGG orthology, pathways, and enzyme commission information.

The abundance of each functional category was determined, and differentially expressed pathways among multiple groups were determined.

### 2.5. Statistical Analysis

Differences in the fundamental physicochemical properties of the substrate, growth and physiological indices of the seedlings, and alpha diversity indices of the bacterial communities were analysed via one-way ANOVA using SPSS 21.0. Significant differences were determined using the Tukey test.

## 3. Results

### 3.1. Differences in Seedling Rhizosphere Nutrient Content among the Different Substrates

The AN, AK, TN, and OM contents of the H treatment group were significantly higher than the corresponding values in the CK and S groups ($p < 0.05$, Table 1). The AK and OM contents were in the order of H > CK > S, and the AN and TN contents were in the order of H > S > CK. The TP and TK contents of the S treatment group were significantly higher than those of CK. The TP content was in the order of S > CK > H, and the TK content was in the order of S > H > CK. The AP content of CK was higher than that of S and H, and it was in the order of CK > H > S. Overall, rhizosphere nutrients were significantly more abundant in seedlings grown in either H or S than in those grown in CK. Therefore, H and S were considered superior substrates, compared to the control, as they provide more nutrients for the growth and development of *Q. variabilis* seedlings and promote plant nutrient uptake.

**Table 1.** Nutrients in the rhizosphere of *Quercus variabilis* seedlings grown in different substrates.

| Physicochemical Indices | H | S | CK |
|---|---|---|---|
| AN ($mg \cdot kg^{-1}$) | $874.71 \pm 7.68^{b}$ | $603.95 \pm 19.76^{a}$ | $589.43 \pm 14.46^{a}$ |
| AP ($mg \cdot kg^{-1}$) | $38.79 \pm 2.56^{b}$ | $12.87 \pm 1.17^{a}$ | $153.18 \pm 2.16^{c}$ |
| AK ($mg \cdot kg^{-1}$) | $1\,208.49 \pm 48.95^{c}$ | $494.11 \pm 7.16^{a}$ | $821.01 \pm 141.70^{b}$ |
| OM (%) | $77.24 \pm 0.57^{b}$ | $49.45 \pm 8.90^{a}$ | $61.11 \pm 2.07^{a}$ |
| TN (%) | $1.76 \pm 0.07^{c}$ | $1.59 \pm 0.04^{b}$ | $0.98 \pm 0.04^{a}$ |
| TP (%) | $0.06 \pm 0.004^{a}$ | $0.10 \pm 0.005^{c}$ | $0.08 \pm 0.001^{b}$ |
| TK (%) | $0.48 \pm 0.02^{b}$ | $1.72 \pm 0.05^{c}$ | $0.37 \pm 0.02^{a}$ |

Note: n = 3. Different letters indicate significant differences at $p < 0.05$. TN: total nitrogen; AN: alkali-hydrolysable nitrogen; TP: total phosphorus; AP: available phosphorus; TK: total potassium; AK: available potassium; OM: organic matter.

### 3.2. Differences in the Growth Indices of Q. variabilis Seedlings Grown in Different Substrates

Significant differences were noted in the plant height, ground diameter, fresh weight, dry weight, fine root length, and fine root surface area of seedlings grown in different substrates ($p < 0.05$, Table 2). The values of fresh and dry root weight of the seedlings were in the order of H > S > CK, whereas the values of fresh and dry shoot weight of the seedlings were in the order of S > H > CK. The fresh weight of roots and shoots of seedlings grown in H and S was significantly higher than that of seedlings grown in CK. The dry weight of roots of seedling grown in H was significantly higher than that of seedlings grown in CK, whereas the dry weight of shoots of seedlings grown in H and S was significantly higher than that of seedlings grown in CK. The plant height and ground diameter of seedlings grown in H were significantly higher than the corresponding values of seedlings grown in CK and S. Plant height was in the order of H > CK > S, and ground diameter was in the order of H > S > CK. Therefore, H and S were determined to be more conducive to OM accumulation in *Q. variabilis* seedlings, resulting in higher dry weight values of roots and shoots. Moreover, *Q. variabilis* seedlings grown in H were taller and showed a greater ground diameter. Additionally, the fine roots of seedlings grown in H and S were significantly longer than those of seedlings grown in CK, and the surface area of fine roots was significantly higher in seedlings grown in H than in those grown in CK. The

results indicate that H significantly promotes the root growth of *Q. variabilis* seedlings, as evidenced by the significantly increased length and surface area of fine roots.

**Table 2.** Growth and physiological indices of *Quercus variabilis* seedlings in different substrates.

| | | H | S | CK |
|---|---|---|---|---|
| Seedling growth indices | Plant height (cm) | 20.00 ± 1.60 $^{b}$ | 16.80 ± 2.69 $^{a}$ | 17.35 ± 2.36 $^{a}$ |
| | Ground diameter (cm) | 0.20 ± 0.03 $^{b}$ | 0.11 ± 0.03 $^{a}$ | 0.09 ± 0.02 $^{a}$ |
| | Root fresh weight (g) | 6.30 ± 1.18 $^{b}$ | 5.78 ± 0.91 $^{b}$ | 4.39 ± 1.44 $^{a}$ |
| | Root dry weight (g) | 2.40 ± 0.53 $^{b}$ | 2.13 ± 0.41 $^{ab}$ | 1.79 ± 0.76 $^{a}$ |
| | Shoot fresh weight (g) | 4.69 ± 1.63 $^{b}$ | 4.87 ± 1.68 $^{b}$ | 2.40 ± 0.51 $^{a}$ |
| | Shoot dry weight (g) | 1.95 ± 0.76 $^{b}$ | 2.07 ± 0.79 $^{b}$ | 0.94 ± 0.22 $^{a}$ |
| | Root water content (%) | 0.62 ± 0.03 $^{a}$ | 0.63 ± 0.02 $^{a}$ | 0.60 ± 0.08 $^{a}$ |
| Thick roots | Root length (cm) | 6.26 ± 3.10 $^{a}$ | 5.42 ± 1.72 $^{a}$ | 4.09 ± 1.46 $^{a}$ |
| | Root surface area ($cm^2$) | 5.41 ± 2.13 $^{a}$ | 5.02 ± 1.65 $^{a}$ | 4.53 ± 1.56 $^{a}$ |
| | Root volume ($cm^3$) | 0.40 ± 0.15 $^{a}$ | 0.39 ± 0.18 $^{a}$ | 0.41 ± 0.16 $^{a}$ |
| Fine roots | Root length (cm) | 137.53 ± 25.49 $^{b}$ | 133.12 ± 52.56 $^{b}$ | 65.17 ± 7.41 $^{a}$ |
| | Root surface area ($cm^2$) | 21.47 ± 3.95 $^{b}$ | 17.47 ± 6.16 $^{ab}$ | 12.93 ± 2.16 $^{a}$ |
| | Root volume ($cm^3$) | 0.40 ± 0.08 $^{a}$ | 0.30 ± 0.14 $^{a}$ | 0.32 ± 0.15 $^{a}$ |
| Physiological indices | Protein content ($mg \cdot g^{-1}$) | 0.020 ± 0.003 $^{ab}$ | 0.025 ± 0.005 $^{b}$ | 0.019 ± 0.004 $^{a}$ |
| | Chlorophyll content ($mg \cdot dm^{-2}$) | 7.61 ± 0.99 $^{b}$ | 7.19 ± 0.81 $^{ab}$ | 5.66 ± 1.29 $^{a}$ |
| | Chlorophyll fluorescence-FV/FM | 0.84 ± 0.02 $^{b}$ | 0.84 ± 0.02 $^{b}$ | 0.82 ± 0.01 $^{a}$ |
| | Net photosynthetic rate ($\mu mol \cdot m^{-2} \cdot s^{-1}$) | 7.18 ± 1.20 $^{a}$ | 8.01 ± 0.64 $^{a}$ | 11.19 ± 2.31 $^{b}$ |
| | Transpiration rate ($mmol \cdot m^{-2} \cdot s^{-1}$) | 1.25 ± 0.46 $^{b}$ | 0.65 ± 0.11 $^{a}$ | 0.75 ± 0.14 $^{a}$ |
| | Stomatal conductance ($mmol \cdot m^{-2} \cdot s^{-1}$) | 50.80 ± 10.48 $^{a}$ | 49.93 ± 9.39 $^{a}$ | 53.10 ± 11.04 $^{a}$ |
| | Intercellular $CO_2$ concentration ($\mu mol \cdot m^{-2} \cdot s^{-1}$) | 343.08 ± 61.95 $^{c}$ | 185.80 ± 33.52 $^{b}$ | 130.20 ± 15.01 $^{a}$ |

Note: n = 10. Different letters indicate significant differences at $p < 0.05$.

### *3.3. Differences in the Physiological Indices of Q. variabilis Seedlings Grown in Different Substrates*

Significant differences were observed in the chlorophyll content, chlorophyll fluorescence, protein content, leaf transpiration rate, intercellular $CO_2$ concentration, and net photosynthetic rate of seedlings grown in different substrates ($p < 0.05$, Table 2). The chlorophyll content of seedlings grown in H was the highest, whereas that of seedlings grown in CK was the lowest. The protein content of seedlings grown in CK and H was significantly lower than that of seedlings grown in S. The leaf transpiration rate and intercellular $CO_2$ concentration of seedlings grown in H were significantly higher than the corresponding values for seedlings grown in CK and S. The chlorophyll fluorescence of seedlings grown in S and H was significantly higher than that of seedlings grown in CK. Conversely, the net photosynthetic rate of seedlings grown in CK was significantly higher than that of seedlings grown in H and S. The results demonstrate that H was favourable for the synthesis of chlorophyll in *Q. variabilis* seedlings, promoted the leaf transpiration rate, and enhanced intercellular $CO_2$ concentration. On the other hand, S was more conducive to protein synthesis in *Q. variabilis* seedlings, although the net photosynthetic rate of seedlings grown in CK was higher.

### *3.4. Differences in the Number of Rhizosphere Bacteria Associated with Q. variabilis Seedlings*

The copy number of rhizosphere bacteria associated with *Q. variabilis* seedlings was in the order of S > CK > H ($p < 0.05$, Table 3). The highest bacterial copy number was observed

in the S treatment group, at $14.56 \times 10^5 \cdot g^{-1}$ substrate, followed by CK, at $7.81 \times 10^5 \cdot g^{-1}$ substrate. The lowest bacterial copy number was found in the H treatment group. The copy number of bacteria associated with seedlings grown in S was 1.86-fold higher than that of bacteria associated with seedlings grown in CK. Therefore, the number of S rhizosphere bacteria supplemented with cork flour was higher compared to the control (CK) substrate.

**Table 3.** Alpha diversity and copy number of rhizosphere bacterial communities associated with *Quercus variabilis* seedlings grown in different substrates.

| | Number of Soil Bacteria (Copies·g$^{-1}$ Substrate) | Richness | Chao1 | Simpson | Shannon-2 |
|---|---|---|---|---|---|
| H | $0.42 \times 10^5$ | 1 852.60 ± 28.79 $^{b}$ | 1 853.76 ± 28.77 $^{b}$ | 0.003 2 ± 0.0002 $^{b}$ | 9.59 ± 0.046 $^{a}$ |
| S | $14.56 \times 10^5$ | 1 902.60 ± 16.06 $^{b}$ | 1 903.56 ± 16.18 $^{b}$ | 0.002 5 ± 0.0001 $^{a}$ | 9.76 ± 0.029 $^{b}$ |
| CK | $7.81 \times 10^5$ | 1 705.60 ± 43.82 $^{a}$ | 1 706.76 ± 43.91 $^{a}$ | 0.002 8 ± 0.0002 $^{a}$ | 9.64 ± 0.067 $^{a}$ |

Note: n = 5. Different letters indicate significant differences at $p < 0.05$.

### *3.5. Differences in the Alpha Diversity of Seedling Rhizosphere Bacteria*

Significant differences were observed in the alpha diversity indices of rhizosphere bacteria associated with seedlings grown in the different substrates ($p < 0.05$, Table 3). The richness and Chao1 indices demonstrated that H and S were significantly higher than CK ($p < 0.05$). The highest bacterial richness and Chao1 indices were recorded in S, followed by H. Overall, the abundance of bacteria in S was greater than that in H and CK. Simpson index values revealed that diversity was significantly higher in the H treatment group compared with CK and S. However, Shannon index values indicate that the S treatment resulted in the greatest diversity; for this reason, we conclude that the rhizosphere bacterial community was more diverse in the S treatment than in CK and H.

### *3.6. Effect of Cork Flour Addition on the Beta Diversity of Seedling Rhizosphere Bacteria*

The results of the principal component analysis of the beta diversity of rhizosphere bacteria associated with seedlings grown in the three substrates are presented in Figure 1. Principal components PC1 and PC2 represent 59.5% and 18.5%, respectively, of the total variation in bacterial communities, with H and S being relatively distant from CK and closer to one another.

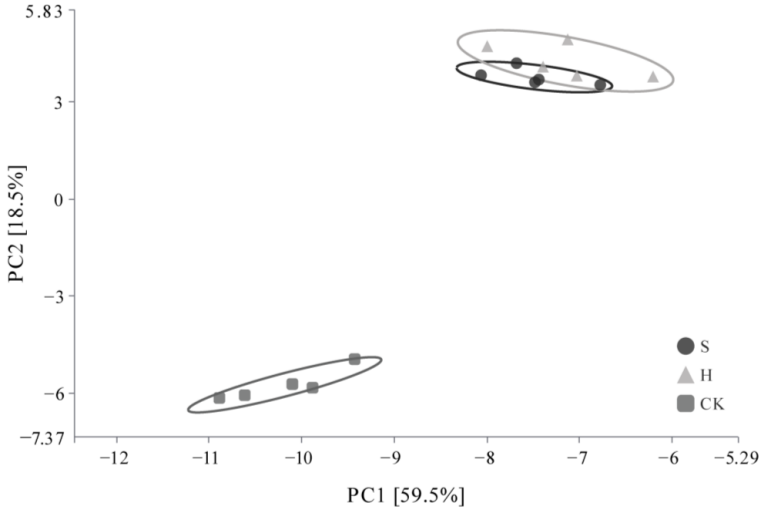


**Figure 1.** Principal component analysis of rhizosphere bacterial communities associated with *Quercus variabilis* seedlings grown in different substrates.

### *3.7. Differences in the Composition of Rhizosphere Bacterial Communities of Q. variabilis Seedlings Grown in Different Substrates*

Analyses of rhizosphere bacterial communities at the phylum, class, order, family, and genus level revealed that communities associated with seedlings grown in H and S substrates were similar but significantly differed from those associated with seedlings grown in the control (CK) substrate ($p < 0.05$, Figure 2). *Proteobacteria*, *Cyanobacteria*, WPS-2, and *Chloroflexi* were significantly more abundant in the rhizosphere of *Q. variabilis* seedlings grown in H and S, whereas *Dependentiae*, *Verrucomicrobia*, *Gemmatimonadetes*, and FBP were more abundant in the rhizosphere of seedlings grown in CK. The abundances of 12 bacterial genera significantly differed among the H, S, and CK treatment groups: *Chujaibacter*, *Hyphomicrobium*, *Methylovirgula*, *Sphingomonas*, *Acidibacter*, *Bordetella*, and *Nocardioides* were significantly more abundant in the rhizosphere of *Q. variabilis* seedlings grown in H and S, whereas *Micropepsis*, *Bryobacter*, *Pseudolabrys*, *Alkanibacter*, and *Dokdonella* were more abundant in the rhizosphere of seedlings grown in CK. At the species level, *Sphingomonas* sp. and *Mesorhizobium* sp. were significantly more abundant in the rhizosphere of *Q. variabilis* seedlings grown in H and S, whereas *Skermanella* sp. was more abundant in the rhizosphere of seedlings grown in CK.

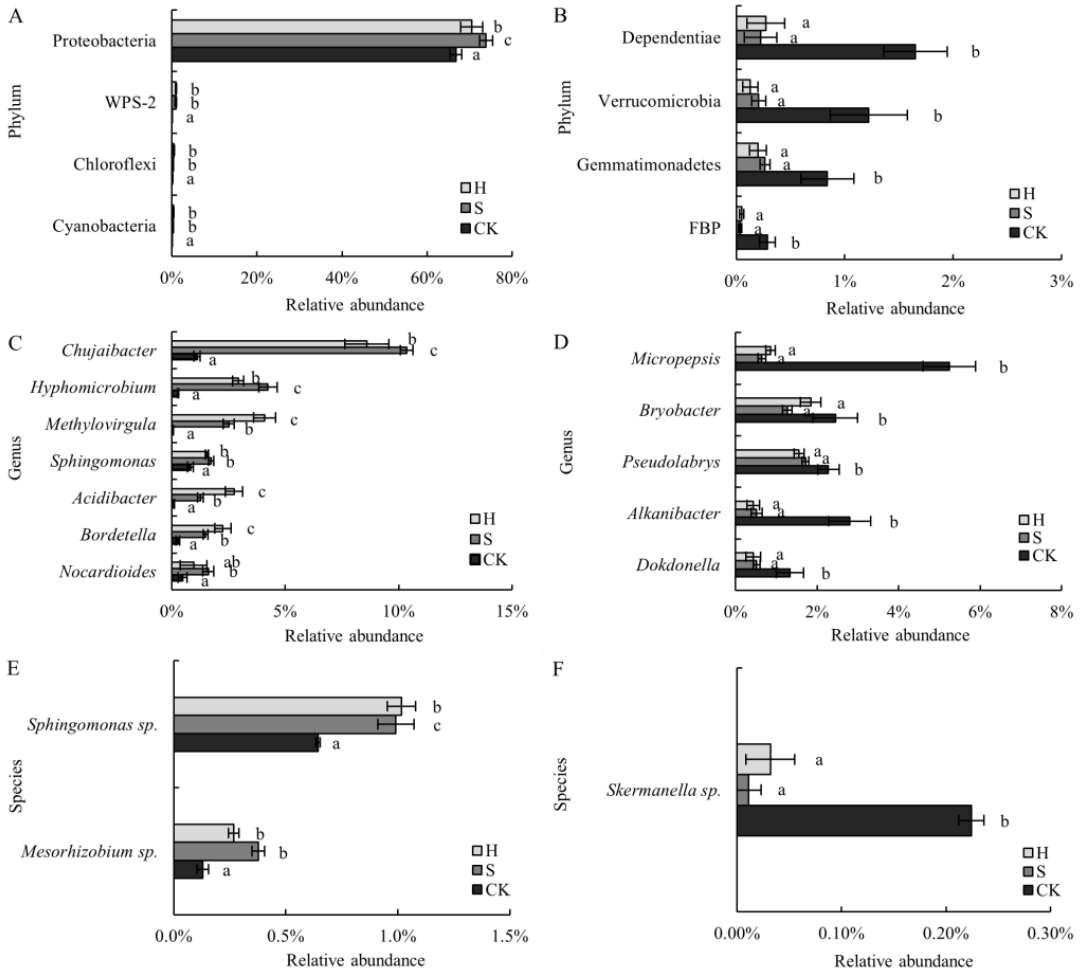


**Figure 2.** Composition of rhizosphere bacterial communities associated with *Q. variabilis* seedlings grown in different substrates: (**A**) S/H > CK at the phylum level; (**B**): S/H < CK at the phylum level; (**C**) S/H > CK at the genus level; (**D**) S/H < CK at the genus level; (**E**) S/H > CK at the species level; (**F**) S/H < CK at the species level. The different letters indicate significant difference ($p < 0.05$).

### *3.8. Functional Analysis of Rhizosphere Bacterial Communities of Q. variabilis Seedlings Grown in Different Substrates*

The results of the KEGG L3 analysis revealed six major functional gene types that significantly differed amongst the three substrates, namely, antibiotics (ansamycin and vancomycin), peptidoglycan biosynthesis, ketone body synthesis and degradation, bacterial chemotaxis, and lipoic acid metabolism (Figure 3). Rhizosphere bacterial communities of *Q. variabilis* seedlings grown in CK were significantly enriched in the following terms: synthesis of antibiotics, peptidoglycan, bacterial chemotaxis, and metabolism of zinc sulphate. In contrast, rhizosphere bacterial communities in the H and S treatments were significantly enriched in the following terms: synthesis and degradation of ketone bodies.

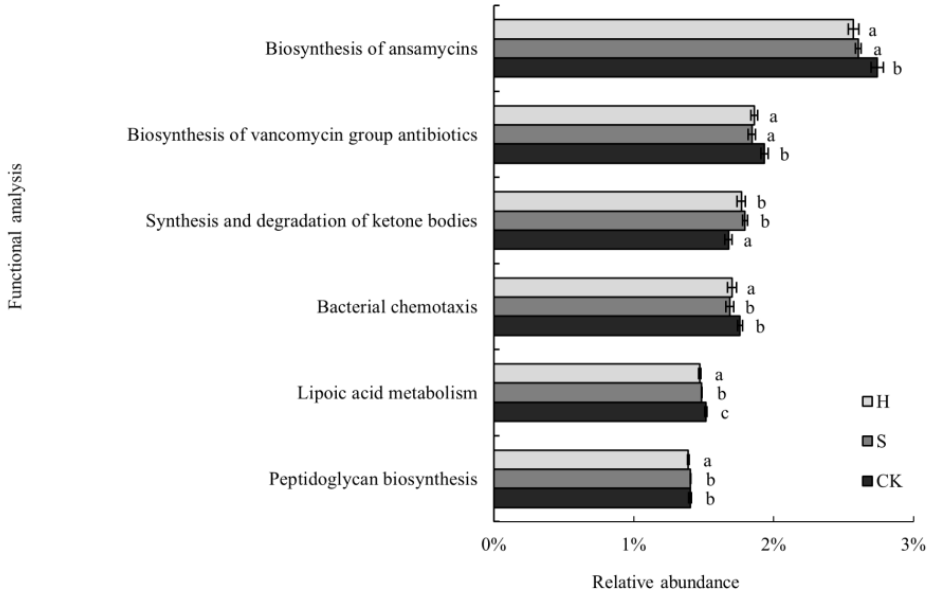


**Figure 3.** Functional analysis of rhizosphere bacterial communities of *Q. variabilis* seedlings grown in different substrates. The different letters indicate significant difference ($p < 0.05$).

## 4. Discussion

### *4.1. Relationship between Differences in Nutrient Contents and Growth and Physiological Indices of Q. variabilis*

In the natural secondary forests of *Q. variabilis*, there are a number of challenges to regeneration [32]. The method of seeding and planting seedlings is key to the regeneration and restoration of *Q. variabilis* stands [33], and seedling substrate is a key factor affecting seedling growth [6,34]. Different substrate materials can have direct or indirect effects on plant growth and development [35], and the correct choice of substrate is critical to the success of the planting system [36]. Grass charcoal ash and perlite have long been considered ideal seedling substrates. Grass charcoal can improve plant nutritional status [37], and perlite is a light inorganic seedling material with excellent water retention capacity [38]. The resilience of container seedlings is often judged based on plant height and ground diameter [39]. Seedling growth primarily depends on the redistribution and transfer of nutrients within the plant, which is closely related to the substrate in which the seedlings are grown [7,40].

In the present study, seedlings grown in the experimental substrate mixes H and S were found to perform better in terms of fresh weight, dry weight, plant height, and ground diameter, possibly because the TP and TK contents of the S substrate were significantly higher than those of the control (CK). The level of soil nutrients (TN, TP, and TK) has been shown to be significantly correlated with seedling growth and plant leaf and root traits to varying degrees [41], and this influences, to varying degrees, plant traits and biomass allocation [42]. Given that phosphorus plays a vital role in promoting nitrogen metabolism and protein synthesis [43], it is not surprising that the foliar protein content of *Q. variabilis*

seedlings grown in S was higher. Furthermore, the contents of AN, AK, TN, and OM were significantly higher in H than in CK and S. Potassium can catalyse various metabolic reactions, affect photosynthesis, and regulate cellular osmosis [44]. Organic matter can improve soil granular structure and enhance soil aeration and water permeability [45]. In addition, the AP content of CK was significantly higher than that of H and S. However, the dry weight of seedlings grown in CK was significantly lower than that of seedlings grown in the other two substrates, which is consistent with previous findings [46]. The biomass of *Q. variabilis* seedlings is negatively correlated with the AP content of substrate; thus, the mass fraction of AP should be reduced when formulating *Q. variabilis* substrates [46]. Therefore, H and S created a better rhizosphere environment for *Q. variabilis* seedling growth. Specifically, H and S may be superior to the control treatment in terms of microbial nitrogen and phosphorus metabolism, given that both substrates increased the rhizosphere nutrient content and promoted nutrient absorption by plants.

### *4.2. Effect of Cork Flour Supplementation on the Diversity of Rhizosphere Bacterial Communities of Q. variabilis Seedlings*

Overall, after 4 months of growth, the rhizosphere bacterial communities of seedlings in H and S substrates were more stable. The significantly higher contents of AN, AK, TN, and OM in the H treatment group and the higher contents of TP and TK in S indicate that H and S could provide additional nutrition for seedling growth. Moreover, the significantly better growth and physiological indices of seedlings grown in cork-flour-supplemented substrates may be directly linked to the greater stability of their rhizosphere bacterial community [47–50]. Plant growth and physiological indices reflect the level of plant growth and development, and rhizosphere microorganisms produce a multifaceted impact on plants. Specifically, rhizosphere microorganisms can improve plant resistance and promote plant growth and development [15]. Moreover, the stability of soil microbial communities is closely related to crop yield, and key microbial bacterial communities (nitrogen-fixing and phosphate-solubilising bacteria) can affect plant productivity [48–50].

*Proteobacteria* comprise the major dominant bacteria, including Gram-negative species, which are typically associated with soil eutrophic status, soil nitrogen cycling, and biological nitrogen fixation, whereas *Verrucomicrobia* are generally associated with poor soil conditions [51–55]. The relative abundance of *Proteobacteria* was significantly higher in H and S treatments compared to the control (CK), whereas the abundance of *Verrucomicrobia* was higher in CK, indicating that the H and S substrates were nutrient- and energy-rich and thus favourable to the growth of *Q. variabilis* seedlings. The results of previous studies have demonstrated that *Hyphomicrobium*, *Sphingomonas*, and *Acidibacter* strains play significant roles in nitrogen fixation, denitrification, phosphate solubilisation [52,54,55], and phosphorus dissolution and can promote plant growth [56]. The high relative abundance of these three genera in the rhizosphere of seedlings grown with H and S indicates that these two substrates could promote the material metabolism and energy cycle of nitrogen, phosphorus, and carbon in rhizosphere bacteria, which is beneficial to plant growth. Specifically, *Hyphomicrobium* (*Hyphomicrobiaceae*) is a genus of denitrifying bacteria that can denitrify and remove phosphorus [56]. Moreover, *Acidibacter* can regulate soil pH and is involved in the carbon cycle of humus decomposition [57]. Further, a metabolic mechanism in *Sphingomonas* (*Sphingomonadaceae*) allows the bacterium to tolerate poor nutrient conditions. Species of this genus are hardy and widely distributed [58] and are associated with nitrogen-fixation, denitrification, and phosphorus-solubilisation functions; furthermore, they have been shown to produce proteases, secrete rhizosphere sugar nutrients, and promote plant nutrient uptake.

In addition, *Sphingomonas* spp. have been determined to be plant-growth-promoting bacteria (PGPB) and promote plant growth through both direct and indirect mechanisms [59]. In the present study, the abundance of *Sphingomonas* was higher in H and S (compared to the control). The results of previous studies have demonstrated that

*Sphingomonas* spp. can promote plant resistance to various pathogens and can effectively help resist pathogenic infections [60], which in turn promotes plant growth [61].

## 5. Conclusions

In the present study, the experimental substrates H and S, which are plant growth substrates supplemented with cork flour at specific ratios, were found to be conducive to the growth and development of *Q. variabilis* seedlings. The S substrate (cork flour/perlite, 1:1) resulted in a higher number and greater diversity of bacteria than was found in either the control treatment (CK, charcoal soil/perlite, 3:2) or the H substrate (charcoal soil/cork flour/perlite, 1:1:2). The substrates supplemented with cork flour (H and S treatments) enhanced the abundance of microbial groups that play important roles in nitrogen fixation, denitrification, and phosphorus dissolution, which likely improved the growth and physiological indicators of *Q. variabilis* seedlings. However, these effects must be further verified using conventional pure culture methods. Overall, substrates supplemented with cork flour can promote the root growth of *Q. variabilis* seedlings, facilitate the synthesis of chlorophyll and protein in *Q. variabilis* seedlings, and increase the abundance and diversity of their rhizosphere bacterial community. Therefore, these substrates can be used as alternatives in the artificial breeding of *Q. variabilis* seedlings. Our findings provide a reference for the artificial breeding and selection of superior *Q. variabilis* seedlings.

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