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The improvement of kinsenoside in wild-imitated cultivation Anoectochilus roxburghii associated with endophytic community

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Abstract

Background

Kinsenoside, a predominant pharmacologically active component in Anoectochilus roxburghii, exhibits great liver protective effect for treating non-alcoholic steatohepatitis, autoimmune hepatitis, alcoholic liver injury, and liver fibrosis, etc. Previous research found that the content of kinsenoside in wild-imitated cultivation A. roxburghii (WC) was similar to that of wild-tending cultivation plants (WP). This manuscript disentangled the relationship between kinsenoside and microbiome of A. roxburghii under wild-imitated environment.

Results

Our research found that endophytic bacteria's diversity and network complexity decreased from WP to WC and tissue-cultured plants (TC). After 3 months of growth in the wild-imitated environment, the microbial composition of TC was significantly differentiated and converged toward WP. Correlation analyses showed that the diversity of bacterial community has a significant positive relationship with the content of kinsenoside, which is the main functional active ingredients in *A. roxburghii*, and *BCP* is the key taxa affecting kinsenoside. In addition, acetylacetyl-CoA reductase, a key enzyme for the synthesis of kinsenoside, was significantly enriched in WC and WP.

Conclusion

we proposed that the medicinal quality of *A. roxburghii* under the wild cultivation imitated method is comparable to wild tending cultivation ones with high kinsenoside content which is likely attributed to the activity of *BCP and* acetylacetyl-CoA. This study provides a basis for the improvement and sustainable utilization of *A. roxburghii* resources.

Introduction

Anoectochilus roxburghii (Wall.) Lindl (Orchidaceae) is a perennial herb widely used in medicine, health care, and beauty industry. Being rich in secondary metabolites like polysaccharides[1], glycosides, organic acids, steroids, flavonoids, and alkaloids[2], this medicinal herb has exhibited significant therapeutic effects in anti-inflammatory[3], anti-oxidation[4], anti-tumor[4], and hepatoprotective protection[5, 6]. Kinsenoside, a predominant pharmacologically active component in *A. roxburghii*, exhibits great liver protective effect for treating non-alcoholic steatohepatitis (NASH), autoimmune hepatitis, alcoholic liver injury, cholestatic liver injury, radiation liver injury, and liver fibrosis, etc. It also has moderate hypoglycemic and hypolipidemic effects, and can repair damaged islet cells and restore insulin secretion ability. All of which made kinsenoside being treated as one of the most important benchmarks for the quality of *A. roxburghii* herb[7].

Anoectochilus roxburghii is mainly distributed in Zhejiang, Fujian, Taiwan, Jiangxi, and Guizhou provinces of China, and has high requirements for environmental conditions[8]. With the low natural reproduction rate, poor environmental adaptability, and overexploitation, wild *A. roxburghii* resources decreased drastically, however, their demand keep growing[9]. Nowadays, most of the *A. roxburghii* seedlings are produced by tissue culture technique, and then cultivated in greenhouse or understory. Tissue culture can resolve the problem of low reproductive rate of orchid plants and shorten seedling breeding period. However, the content of secondary metabolites from cultivated *A. roxburghii* is lower remarkably than those of wild ones, which profoundly influence their medicinal applications. Therefore, finding suitable cultivation methods to improve the quality of *A. roxburghii* herb is urgent. Under-forest cultivation provides a suitable environment for *A. roxburghii* such as high humidity, sufficient oxygen, low light intensity and small temperature difference between day and night (which is shade-tolerant). This cultivation pattern mimics the natural condition of *A. roxburghii* which primarily contributes to the accumulation of metabolites. Previous studies have shown that the content of polysaccharides and flavonoids of *A. roxburghii* after understory planting is significantly higher than that in greenhouses and tissue-cultivated seedlings[10, 11]. In addition, the wild cultivation imitated method not only improves the medicinal quality, but makes full use of forest resources and promotes the sustainable development of under forest economy.

The plant microbiome is an essential determinant of plant health and productivity[12]. The microbiome of medicinal plants may directly impact the metabolism of the host, and thus could influence the efficacy of herbal medicine[13]. Two main stratagems have been proposed on microbes influence the production and accumulation of bioactive ingredients in medicinal plants. Firstly, endophytes associated with medicinal plants have the potential capacity of synthesizing host-like bioactive chemical ingredients *in vitro*[14]. For example, *Taxomyces andreanae*, an antitumor active components producer, was isolated from *Taxus brevifolian* Nutt and could produce paclitaxel and taxanes in semi-synthetic culture medium[15]. Secondly, some endophytes contributed to the synthesis of active ingredients by host plants[16]. Studies showed that endophytic bacterium-triggered reactive oxygen species can directly oxidize oxygen-free sesquiterpenoids and increase the oxygenous sesquiterpenoids content and diversity in *Atractylodes lancea*[17, 18]. Orchid has a long history of mycorrhizal symbiosis, and forms mutual relationship with phylogenetically and ecologically diverse fungi. More than 200 endophytic fungi have been isolated from *A. roxburghii* and *Ludisia* orchids[8]. Two strains were found to significantly increase the biomass of *A. roxburghii* and promote the biosynthesis and accumulation of active components after plant-microbe co-cultivation[8, 19].

Microorganisms play a crucial role in the production and accumulation of active components in medicinal plants. However, whether there are interactions between *A. roxburghii* and microbes to promote the production of the secondary metabolite remains largely to unravel. Previous research has revealed that wild cultivation imitated method can significantly improve the quality and phenotype of *A. roxburghii*. However, whether this quality optimization is accompanied by the role of microorganisms, and how these microbial communities play a regulatory role, need further investigation. In this study, the content of major bioactive components of *A. roxburghii* under different growth patterns were measured by microplate reader and high-performance liquid chromatography (HPLC) system, and their endophytic diversity, composition and function were investigated through high-throughput sequencing. By uncovering the interaction between microbes and the secondary metabolism of *A. roxburghii* under different growth patterns, the aim of this study was to answer why wild cultivation imitated method could improve the quality of herbs from microbiome perspective.

Results

Significant difference of active ingredient contents under different growth patterns

Significant difference of active ingredient contents of *A. roxburghii* under different growth patterns were detected by ANOVA and LSD (Fig. 1). WP had the highest content of kinsenoside (292.4 mg/g), which was significantly different from that of TC (144.4 mg/g), but similar to WC (287.3 mg/g) (Fig. 1B). The content of total flavonoids, total polyphenols, and total polysaccharides were the highest in WC. The difference of total polyphenol content between TC and WC was non-significant, yet, the content of total flavonoids and total polysaccharides were significantly different among the three growth methods (Fig. 1B).

Microbial diversity and community composition under different growth patterns

Endophytic diversity analysis showed that different growth patterns exhibited variations in microbe community composition. Chao1 richness and Shannon diversity index were the highest in WP, and the lowest in TC, which is the same as Simpson and ACE (Table S1). When comparing different compartments, the highest bacteria Chao1 and Shannon diversity indexes were found in stems of WP, in leaves of WC, and in roots of TC. The highest Chao1 richness and Shannon diversity indexes of fungi were found in leaves of WP, WC and TC (Fig. 1C, Fig.S2, Table S1).

Nonmetric multidimensional scaling (NMDS) ordination based on Bray-Curtis distance matrices showed significant differences in species composition between different patterns, whether of fungi or bacteria. The difference was the most significant in the leaf under different growth modes (Fig. 1D). PERMANOVA analysis showed that different patterns had significant effects on the microbial communities in different compartments, with the most potent effect on leaf bacteria at 63.1% and the weakest effect on stem fungi at 22.2% (Table 1).

Table 1
Effects of different growth patterns in different compartments on the assembly of the microbiome based on PERMANOVA.

Samples	Bacteria		Fungi	
	Variation (R^2)	P	Variation (R^2)	P
Plant (n = 45)	0.429	0.001	0.222	0.001
Root (n = 15)	0.602	0.001	0.43	0.001
Stem (n = 15)	0.544	0.001	0.299	0.001
Leaf (n = 15)	0.631	0.001	0.419	0.001
Soil (n = 20)	0.434	0.001	0.425	0.001
Note: Soil include rhizosphere soil and bulk soils.				

Taxonomic classification revealed that bacterial and fungal communities in plant organs differed significantly under different patterns (Fig. 2). We observed that the endophytic bacteria in TC were largely composed of *Actinobacteriota* (95.57%), while WC and WP were mainly composed of *Proteobacteria* (WC: 87.48%, WP: 69.55%). The endophytic fungi of TC were mostly composed of *Basidiomycota* (66.18%), *Ascomycota* for WC (85.05%), and *Basidiomycota* (49.69%) and *Ascomycota* (44.22%) in WP (Table S2). It is worth noting that the bacterial community structure is the simplest in TC, which is mainly composed of *Mycobacterium* (92.02%) with the majority derived from leaves (98.61%). On the contrary, WC (5.57%) and WP (7.18%) had extremely low relative abundances of *Mycobacteria* throughout the plant. *Massilia* and *Pseudomonas* accounted for 48.52% of total bacterial endophytes in WC. *BCP* was dominant in WP (26.88%), but its relative abundance gradually decreased from roots (50.84%) to stems (27.54%) and leaves (6.72%), exhibiting a certain degree of compartment/organic specificity (Fig. 2A, Table S2). *Apiotrichum* (25.50%) was the dominant fungus community in TC. The fungal community of WC was relatively simple, with 66.96% composed of *Fusarium*. However, its relative abundance gradually decreased from roots (92.36%) to stems (43.44%) and leaves (7.48%), and *Hannaella* (24.73%) replaced the dominant position of *Fusarium* in leaves. *Thanatephorus* (20.60%) was the predominant fungus in WP, with the majority (61.25%) occupying the stem organ (Fig. 2B, Table S2).

Network analyses and source tracking of endophyte communities of A. roxburghii

To further characterize the impact of growth patterns on microbial communities, especially on the endosphere, the co-occurrence networks of bacteria and fungi under different patterns were constructed (Fig. 3, Table 2). Specifically, bacterial network complexity increased from TC (The average degree: 8.24) to WC (14.69), with the highest value detected in WP (29.59) (Fig. 3A). However, in fungi, TC (26.55) possessed the highest network complexity (Fig. 3B). The leaf endosphere of WP had the highest network complexity among bacterial taxa (Fig.S3A), while the stem endosphere of TC had the highest network complexity among fungal taxa (Fig.S3B). By analyzing the bacterial symbiosis mode under different growth patterns, 23 modules were found. Module 1 and Module 2 were dominant in TC and WC. Among them, *Rhodococcus, Ralstonia*, and *Pseudomonas* were the main bacterial taxa in Module 1 and Module 2 of TC. *Massilia, Pseudomonas*, and *Flavobacterium* were major in Modules 1 and 2 of WC. Module 3 was dominant in WP. The relative abundances of *Burkholderia-Caballeronia-Paraburkholderia* (BCP) and *Acidothermus* were the highest in Module 3 of WP (Table S3). By analyzing the fungal symbiosis patterns of different growth patterns, there were 18 Modules for fungi. TC was dominated by Module 5 and Module 6, and the relative abundances of *Aspergillus* and *Rhodotorula* were the highest in these two Modules than any other endophytic fungi. WC was dominated by Module 1 which was mainly composed of *Hannaella*, *Camptophora*, and *Neoascochya*. WP was dominated by Module 3 with the highest relative abundance detected in *Devriesia* (Table S4).

Table 2
Bacterial and fungi co-occurrence network characteristics in each compartment.

	Samples	Niche	Node	Positive edge	Negative edge	Average degree	Modularity[a]	Average clustering coefficient[b]	Average path distance[c]	Hub node[d]
Bacteria	Plant	TC	221	904	7	8.24	0.676	0.602	5.32	0
		WC	579	4234	18	14.69	0.705	0.545	5.36	0
		WP	639	9437	18	29.59	0.489	0.56	4.37	54
	Root	TC	192	831	6	8.72	0.6	0.602	4.83	0
		WC	205	1103	7	10.83	0.515	0.57	4.89	0
		WP	285	2406	18	17.01	0.511	0.555	3.65	2
	Stem	TC	73	162	3	4.52	0.658	0.576	4.36	0
		WC	306	1528	6	10.03	0.667	0.545	4.78	0
		WP	396	6041	15	30.59	0.386	0.577	3.39	77
	Leaf	TC	27	37	0	2.74	0.704	0.878	1.35	0
		WC	280	1436	6	10.3	0.614	0.513	4.62	0
		WP	349	7065	9	40.539	0.311	0.586	2.8	97
	Soil[e]	WC	326	2869	16	17.7	0.555	0.574	4.776	0
		WP	288	2393	1	16.63	0.419	0.582	3.27	8
Fungi	Plant	TC	337	4550	4	26.55	0.566	0.811	4.549	49
		WC	311	1019	5	6.59	0.694	0.464	6.088	0
		WP	489	3090	6	12.66	0.642	0.46	4.311	2
	Root	TC	133	1817	0	27.33	0.274	0.886	2.711	0
		WC	114	255	5	4.56	0.633	0.479	3.753	0
		WP	261	1142	6	8.8	0.675	0.444	4.325	2
	Stem	TC	153	2301	4	30.13	0.229	0.817	2.136	42
		WC	159	431	5	5.48	0.631	0.509	3.663	0
		WP	234	979	6	8.42	0.657	0.484	4.175	0
	Leaf	TC	108	546	0	10.11	0.652	0.784	2.499	0
		WC	186	642	1	6.91	0.578	0.454	3.422	0
		WP	310	2122	1	13.7	0.525	0.462	3.794	2
	Soil[e]	WC	130	325	1	5.02	0.603	0.462	5.252	0
		WP	316	1478	2	9.37	0.678	0.455	4.301	0

- a Degree of nodes tending to differentiate into different network modules.
- b Degree of nodes tending to cluster together.
- c Network path distance is the length of the shortest path between two nodes within the network.
- d Hub node is defined as a node with high values of degree (> 60) and closeness centrality (> 0.3) in the network.
- e Soil include Rhizosphere soil and Bulk soil.

Source-tracking analysis was conducted to identify the potential sources of microbial communities in different compartments of *A. roxburghii*. Almost all the endophytic bacteria (94%) in leaves of TC came from stems. For WC, 77% of rhizosphere bacteria were derived from bulk soils, and then transferred gradually to roots (31%), stems (35%) and leaves (30%). Similarly, most of the endophytic fungi in roots were derived from the rhizosphere (79%), and then transferred to stems (20%) and leaves (21%). For WP, the majority of endophytic bacteria in roots were derived from rhizosphere (54%) and then transferred to stems (47%) and leaves (20%), but endophytic fungi of the plant were rarely derived from the soil fungi community (Fig. 4).

Keystone taxa, biomarker taxa, and potential function of microbiome under different growth patterns

To better characterize the host effect under different growth patterns, keystone taxa and biomarker taxa of *A. roxburghii* were identified at genus level. The keystone bacterial taxa in TC were mainly *Pseudomonas*, *Massilia*, and *BCP*. WC were largely composed of *Pseudomonas*, *Comamonas*, and *Flavobacterium*. *Kutzneria*, *Mycobacterium*, and *BCP* were the keystone taxa in WP (Table S5). For fungi, the keystone taxa of TC included *Apiotrichum*, *Aspergillus*, and *Epicoccum.* The keystone taxa of WC were mainly composed of *Hannaella*, *Mortierella*, and *Cladosporium*. *Cladophialophora. Devriesia* and *Cyphellophor*a were the keystone taxa in WP (Table S6).

Random forest was used to calculate the relative importance of microbiota. Representative microbial combinations can be treated as biomarkers for distinguishing different growth methods. *Pseudomonas*, *Stenotrophomonas*, *Massilia*, and *Mycobacterium* were identified as bacterial biomarkers for distinguishing three growth patterns, and *Exophiala*, *Acremonium*, and *Fusarium* as fungal biomarkers (Fig.S6, S7). Coincidently, LEfSe analysis indicated that *Mycobacterium* was also an important biomarker in TC, *Flavobacterium*, *Pseudomonas*, *Massilia* were the biomarkers with statistical differences in WC. And *BCP*, *Bradyrhizobium* and *Acidothermus* as important biomarkers in WP. For the fungal taxa, LEfSe analysis indicated *Apiotrichum*, *Cryptococcus* and *Wickerhamomyces* were important biomarkers in TC, *Fusarium*, *Hannaella* were the important biomarkers with statistical differences in WC;*Thanatephorus*, *Cladophialophora* and *Devriesia* were the biomarkers in WP (Table S7).

Correlation between endophytic community and active components of A. roxburghii in different growth patterns

By analyzing correlation between the diversity of endophyte community and the content of active ingredients, the diversity of the bacterial community has a significant positive correlation with the kinsenoside, and a significant negative correlation with polysaccharide (Fig. 5). However, no such significant correlation was found between fungal community and active component contents (Table S9). By defining the keystone taxa and biomarker taxa as the core communities, the correlation between the core communities and active ingredient contents under different growth patterns was analyzed. 75.02% (WC) and 92.27% (WP) of endophytic bacteria were positively correlated with kinsenoside, and almost all fungi (WC: 96.30%, WP: 99.88%) was positively correlated with kinsenoside. *Proteobacteria* enriched in WC was positively correlated with the content of kinsenoside. However, endophytes from TC (bacteria: 98.78%, fungi: 89.02%) were negatively correlated with the content of kinsenoside. Specifically, *Mycobacterium*, *Cutaneotrichosporon, Aspergillus*, and *Mrakia* accounted for a large proportion of TC and were negatively correlated with the content of kinsenoside, but these endophytes were extremely low in WC and WP. *BCP*, mainly found in WC and WP, was positively correlated with kinsenoside content.

After functional annotation by PICRUSt2, 391 traits derived from bacterial database were obtained at KEGG level 3 (Table S9) with little difference among the three growth patterns (Fig.S4). The top five functional traits are metabolic pathways (Pathway ID: ko01100), biosynthesis of secondary metabolites (ko01110), microbial metabolism in diverse environments (ko01120), biosynthesis of antibiotics (ko01130), and carbon metabolism (ko01200). While a total of 904 traits based on fungal sequences were obtained. The relative abundance of some synthetase including Adenosinetriphosphatase, Glucan1,4-alpha-glucosidase, and L-arabinose isomerase was significantly higher in WC than in TC and WP (Fig.S5, Table S10). Notably, acetylacetyl-CoA reductase, a key enzyme for the synthesis of kinsenoside, was significantly enriched in WC and WP. The abundance of acetylacetyl-CoA reductase was significantly higher in WC and WP than TC(Fig. 5). Moreover, the abundance of *Ralstonia eutrophus* that can produce acetyl-CoA was the highest in WC.

Discussion

Anoectochilus roxburghii has more advantages in acquiring keystone microbiota and adapting to the environment after being transplanted into the wild.

We found that when TC seedlings were cultivated in the wild environment for three months (WC), the endophyte community largely restructured. Among the keystone microbiota, apart from the 5 bacteria shared among TC, WC, and WT, 17 bacteria were additionally shared between WC and WP, but no bacteria were shared between TC and WP. For endophytic fungi, 19 bacteria were specifically shared between WC and WP, but only 3 shared between TC and WP. It has been reported that transplantation can lead to the recombination of host endophytes, that is, the greater the environmental difference, the higher the recombination caused by transplanting[20]. In this study, the tissue cultured TC has a relatively simple microbiota, WC and WP have more common and keystone microbiome than TC, indicating that wild environment is more conducive to obtaining keystone taxa. Previous studies have shown that the higher the specialization of microenvironment, the stronger the selectivity and enrichment of microorganisms[18]. After transplanting, the diversity of endophytes was significantly higher than before. Some of the predominant strains were also altered. *Mycobacteria*, a dominant taxon in TC (95.55%), decreased significantly after transplanting and were replaced by *Proteobacteria*. And a large number of *Fusarium* was accumulated in WC.

The SourceTracker program was utilized to track the source of endophytic microbial communities under different growth patterns. Most of the endophytes in the leaves and roots of TC come from the stems, which may be because the stems were used as the materials for tissue culture, so the endophytes in TC are almost inherited from the parental plants. In WC and WP, a relative part of the root endophytic bacteria come from the rhizosphere soil, and the endophytes in the roots are gradually transferred to the stems, while a part of the stems are transferred to the leaves. A previous study had shown that the soil is the main source of microbial selection for plant, which can colonize plant compartments through roots and other external forces[21]. After host filtering, certain microbial taxa can be enriched in different plant niches. These endophytes actively selected by the host have physiological plasticity that thrive in different plant compartments and may play important functional roles[22]. Interestingly, we also found that the main source of endophytic fungi in WC is the rhizosphere soil. Compared with TC and WP, fungal community have more "communication" in WC, so we speculate that some of the key taxa fungi in WC may be obtained from the soil environment through roots.

Transplantation not only affects the endophyte community structure, but also the interrelationships among microbes. Network properties such as modularity derived from network co-occurrence analysis enable prediction of microbial relationships that are critical to community stability[23]. This study shows that the stability of endophytes in *A. roxburghii* can be improved after transplanting. Firstly, modularity reflects the shared ecological functions[24] and spatial division[25] of microbes, quantifies the strength of mutual effect, thereby largely affecting the stability of microbial communities[26]. High modularity helps maintain a stable community, lower the impact of missing taxa on its own modules as well as on the remaining network[25, 27]. In present study, transplanting increased the modularity of the endophytic community network of *A. roxburghii*, suggesting that transplanting may improve microbial community stability. Second, we also found that the bacterial network complexity of WC increased significantly after transplantation compared with TC. It has been shown that complex networks and excellent connectivity are more resistant to environmental perturbations than simple networks with low connectivity[28]. In this sense, endophytic bacteria from wild-originated *A. roxburghii* were more resilient to environmental stress, which is not the case of endophytic fungi. Further studies are needed to confirm this observation.

The improvement of kinsenoside in A. roxburghii is associated with the endophytic communities

The study of microbial community structure and functional capabilities associated with medicinal plants has attracted great attentions[29, 30]. Kinsenoside, an important quality indicator of *A. roxburghii*, has prominent pharmacological effects such as anti-inflammatory, hepatoprotective, and anti-hyperglycemia[31]. Among the three growth patterns, the content of kinsenoside in WP was significantly higher than TC, and comparable to WP. *BCP*, the dominant microbe in WP and WC, was positively correlated with kinsenoside content. In recent years, *Burkholderia* has been widely applied as plant growth-promoting bacteria and biological fertilizers[32]. The *Burkholderia-Caballeronia-Paraburkholderia* group was proposed to be an assemblage of ginsenoside-

enrichment bacteria, which could promote plant growth, increase plant resistance, and reduce the toxicity of glyphosate, benzoic and phthalic acids[33–35]. In this study, *Burkholderia* strains may have great potential as biological fertilizer to promote the plant growth and accumulation of active ingredient content in *A. roxburghii*. *Fusarium* was the dominant endophytic fungi in WC. Studies have shown that Fusarium is a treasure source of active natural products and abundant in secondary metabolites[36]. Although *Fusarium* has been proved to be the pathogen causing many fusarium wilt diseases[37, 38], it has also been reported that *Fusarium* can induce jasmonic acid (JA) signaling pathway[39], regulate the expression of defense related genes, and promote the production of secondary metabolites. In order to resist *Fusarium* oxysporum, tomatoes produce defense related enzymes, phenols, flavonoids and phenolic acids[40] through the crosstalk of salicylic acid (SA), JA, nitric oxide and reactive oxygen species (ROS). The presence of *Fusarium* has also been proved to activate important modifications in host plant metabolism and affect the levels of sugars, polyols and some amino acids[41]. Therefore, a large amount of *Fusarium* accumulated in WC may increase the content of total flavonoids in plants by activating the host metabolic pathway.

Acetoacetyl CoA is reduced to R-3-hydroxybutyryl CoA by an acetoacetyl CoA reductase[42]. 3-hydroxybutyryl CoA the precursor substance that catalyzes the synthesis of intermediate 3,4-dihydroxybutyric acid (3,4-DHBA) during the synthesis of kinsenoside [43,44]. That is acetyl-CoA reductase is the key factor in the synthesis of kinsenoside. Notably, acetylacetyl-CoA is not only present in plants, some microorganisms such as *Ralstonia eutrophus* and *Azohydromonas* have been confirmed to produce acetylacetyl-CoA[45]. The acetylacetyl CoA reductase of *R. europhus* and *Azohydomonas* can catalyze its production of secondary metabolites in tobacco, oilseed rape (*Brassica napus*), and cotton (*Gossypium spp.*), known as polyhydroxybutyrate (PHB)[42]. The results showed that the abundance of *R. eutrophus* that can produce acetyl-CoA was the highest in WC, and the abundance of acetylacetyl-CoA reductase was significantly higher in WC and WP than in TC, and the content of kinsenoside was also significantly higher in WC and WP than in TC, suggesting the microbiome in WC and WP may be enriched in functions related to kinsenoside metabolism, and thus contribute additional metabolic capabilities to those encoded in the genome of the host plant.

Keystone microbiota and their ecological function variation in different growth patterns

Regardless of microbial abundance, the keystone taxa play an essential role in the structure and ecosystem function of microbiome[22, 46]. Keystone taxa (bacteria: *Pseudomonas, Massilia, BCP*, *Methylobacterium-Methylorubrum*, and *Roseiarcus*; fungi: *Apiotrichum, Trichomerium, Mortierella*, and *Cladosporium*) were found in all three growth patterns. *Pseudomonas, Fusarium*, and *Cladosporium* are also considered to be the core microbiota in plants such as barley, rice, and Arabidopsis[47, 48]. *Pseudomonas* are abundantly enriched in the roots of WC, but their abundance decreased continuously from roots to stems and leaves. *Pseudomonas* can inhibit phytopathogens through antagonism and competition, promoting biocontrol and plant growth[47, 49]. Existing studies have confirmed that symbiotic *Pseudomonas* can protect Arabidopsis from pathogenic *Pseudomonas* by reducing its proliferation within the plant[50, 51]. *BCP* and *Pseudomonas* are considered to be plant growth-promoting bacteria[52], and inoculation of these two bacteria into continuously tilled soils can significantly alleviate negative plant-soil feedback[53, 54]. We therefore speculate that Pseudomonas and *BCP*, which are abundantly enriched in WP and WC, respectively, may play an important growth-promoting role in *A. roxburghi.*

The main taxa in the microbial community are also considered as potential key taxa[55]. *Fusarium*, as the main taxa in WC, is abundant in the root of WC but less in TC and WP. Although, members of the genus *Fusarium* have been commonly identified as plant pathogens[37]. But a growing body of researches is showing that the same *Fusarium* species could possibly be either pathogenic or nonpathogenic species according to the different crop species and soil environments[56–58]. *Fusarium* most likely play roles as antagonists of sweet potato disease development and may play an important role in its cultivation[59]. Sometimes *Fusarium* can cause host plants to regulate oxidative balance and activation of the salicylic and jasmonic acid signaling pathways to increase abiotic as well as biotic stress resistance, and even may enhance plant growth[60, 61]. *Fusarium* sp. HGUP190163, isolated from *A. roxburghii*, had antibacterial activity[62] in vitro and vivo[63]. In contrast to tissue culture, *A. roxburghii* recruited a great number of *Fusarium* might to protect plants from pathogens after transplanting to the wild.

Conclusion

Anoectochilus roxburghii is a rare and precious Chinese medicinal herb with various pharmacological activities. The wild resource of *A. roxburghii* is scarce and far from meeting the increasing market demands. Moreover, the quality of tissue-cultured *A. roxburghii* remains controversial partly due to the low effective component content. The effect of wild cultivation imitated method was evaluated concerning the endophyte community and active ingredient contents of *A. roxburghii* via comparing to tissue-cultured and wild tending cultivation plants. Our results show that endophytic bacterial diversity and network complexity decrease from WP to WC and TC. After the TC seedlings being transplanted to the wild (WC), it has more advantages in acquiring core communities and adapting to the surrounding environment. Notably, *Mycobacterium*, which dominates in TC, is almost absent in WC after transplantation. In addition, the eutrophication bacteria of *Ralstonia*, which produced acetyl-CoA, had the highest abundance in WC, and the bacteria *BCP* is abundantly enriched in the roots and rhizosphere soils of WP and is the key taxa affecting the accumulation of kinsenoside. *Pseudomonas* and *Fusarium* are abundant in WC and play a crucial role in the biological control and growth of *A. roxburghii*. This study showed firstly direct evidence of a close relationship between endophytes and the content of active ingredients in *A. roxburghii*. Future research focusing on the isolation and screening of endophytes to improve the content of active ingredients in *A. roxburghii* through beneficial interactions between microorganisms will be conducted.

Materials

Experimental design

Anoectochilus roxburghii under wild cultivation imitated method (WC), wild tending cultivation plants (WP), as well as tissue-cultured plants (TC) were collected from Longyan, Fujian Province, China, in November 2020 (Fig.S1, 24°43'58"N, 116°50'39"E). The germplasm resources of WC and TC were identical and provided by Gelv Company (Fujian, China), which were tissue-cultivated for three months. After that, some plants were transplanted to the wild-imitated environment (Fig. 1A) where the WP grew on August 12th, 2020, and some were kept on tissue cultivation.

Sample collection and treatment

After three months of treatment, 100 plants were randomly collected in each growth patterns on November 20th, 2020. Twenty plants were used for microbe investigation with their plant surface sterilized and divided into five groups, and each group was further separated into three compartments (roots, stems, leaves)[21, 64]. The root samples were rinsed three times with phosphate buffer (pH 7.2) in an ultrasonic cleaner and the sediments were treated as rhizosphere soil after centrifugation. Bulk soils were collected from the 0–15 cm soil layer 0–10 cm away from the plant. No soils were collected from TC. Finally, a total of 65 samples were collected in this study. All the samples were stored at -80°C for microbial sequencing.

Active ingredients determination

Another 80 plants were oven-dried at 50°C for 12 h and crushed into powder for active ingredient determination. The contents of kinsenoside contents were detected based on High Performance Liquid Chromatography-Evaporative Light Scattering Detector (HPLC-ELSD) by Waters (Shanghai, China) (see supplementary methods 2–5 for details regarding the protocol).

DNA extraction and high-throughput sequencing

The total DNA of roots, stems, leaves, and soils were extracted using the PowerSoil DNA Isolation Kit (QIAGEN, Shanghai, China) according to the manufacturer's instructions. DNA quantity was detected using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Approximately 10 ng DNA was used as the template. Primers 799F (5'-AACMGGATTAGATACCCKG-3') and 1193R (5'-ACGTCATCCCCACCTTCC-3'), primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') were used to amplify the V5-V7 region of 16S rRNA gene and ITS region,

respectively[65]. Amplicon libraries and DNA sequencing were conducted using an Illumina MiSeq PE300 platform by Shanghai Majorbio Bio-pharm Technology Co., Ltd., following the manufacturer's protocols.

Sequence analysis

Bioinformatics processing of 16S rRNA and ITS sequences were based on previous studies[66–70]. Briefly, the PE reads samples were split after MiSeq. The double-ended readings were first controlled and filtered according to the quality. The optimized data was obtained by overlapping the relationship between the double-ended readings. To obtain the representative sequence and abundance information of ASV (Amplicon Sequence Variant), the date was optimized by the sequential denoising method (DADA2)[71]. The species were annotated with the Bayesian algorithm[72]. The confidence analysis threshold was 0.7 and the databases were SILVA (v138) and UNITE (v8.0) for bacteria and fungi, respectively[73]. 4307779 bacterial sequence and 4045935 fungal sequence were optimized from 65 samples, and further divided into 9358 bacteria ASVs and 8973 fungal ASVs. Singletons and ASVs assigned as chloroplasts and mitochondria sequences were removed from the data before downstream analysis. The diversity of bacteria and fungi were calculated in QIIME2[74].

Statistical analysis

Analysis of Variance (ANOVA) was performed on the content of active ingredients of *A. roxburghii* in different growth patterns using IBM SPSS Statistics (version 26.0)[75]. Alpha diversity indices (e.g., Shannon index and Chao1 index) were calculated using QIIME[76]. The beta diversity was assessed by computing the Bray-Curtis distance matrix and then ordinated using non-metric multidimensional scaling (NMDS). PERMANOVA was used to analyze the significance of differences between different growth patterns at different tissue locations based on the Bray-Curtis distance. Network analysis was performed using routine CoNet in Cytoscape v.3.8.4 and visualization in Gephi[77]. The RANODMFOREST package in R was conducted to identify the most important dominant taxa in three growth patterns[78]. And the linear discriminant analysis effect size (LEfSe) was also applied (Wilcoxon P-value < 0.05, logarithmic linear discriminant analysis (LDA) score > 2) to identify the biomarkers of different growth patterns[79]. The SourceTracker program was used to estimate potential sources of microorganisms in different plant compartments[70]. The FUNGuild database[80] and the FAPROTX database[81] were used to predict the functions of bacteria and fungi, respectively. Spearman correlation coefficient between keystone taxa and the four active components were calculated in SPSS, and correlation network were generated in Cytoscape (Spearman, $P < 0.05$)[82].

Declarations

Ethics approval and Consent to participate

Not applicable.

Consent for publication

All authors have given consent for publication.

Availability of data and materials

Raw 16S rRNA gene amplicon sequence data have been deposited in the Sequence Read Archive under BioProject PRJNA772090. Source data are provided with this paper.

Competing interests

The authors declare no competing interests.

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Author contributions

Y.Z., and X.L., conducted the experiment, analyzed the data, and wrote the manuscript. L.L., S.X., and X.S., conducted the SourceTracker and network analyses. Z.Z., conducted the experiment. H.G., and Q.S., conceived the experiment and revised the manuscript. All authors read and approved the final manuscript.

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Figures

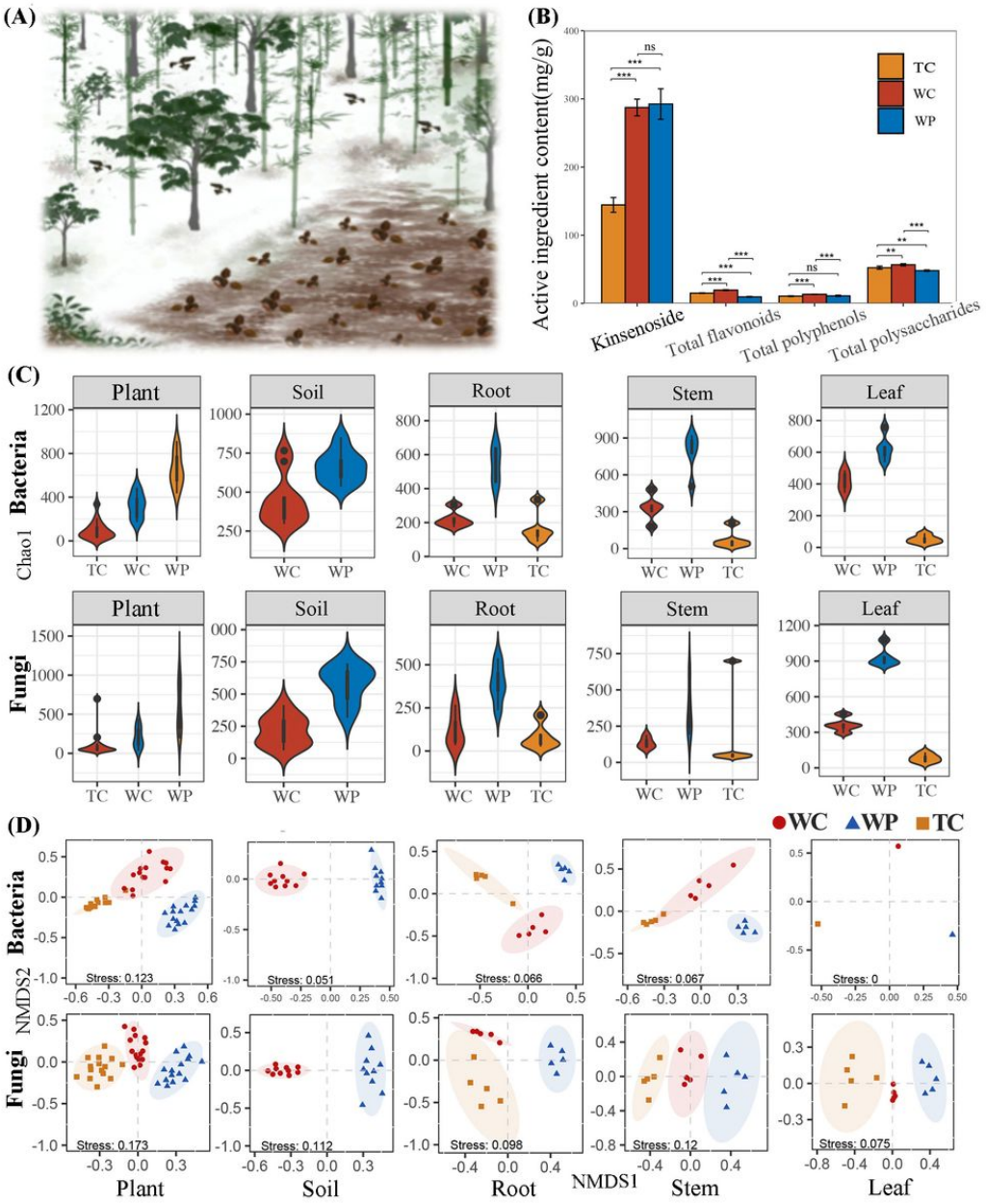


Figure 1

The growth pattern, contents of four active ingredients, diversity and community structure of *A. roxburghii* under different growth patterns. **(A)** Schematic diagram depicting the growth environment of wild and imitating wild cultivation of *A. roxburghii.* **(B)** The contents of four active ingredients (kinsenoside, total flavonoids, total polyphenols, and total polysaccharides) of *A. roxburghii*under different growth patterns. * P < 0.05, **P < 0.01. ***P< 0.001(LSD). **(C)** Alpha-diversity (Chao1) of bacterial and fungal communities in plant compartments (leaf, stem and root) and soils (rhizosphere and bulk soils) under different growth patterns. **(D)** Nonmetric multidimensional scaling (NMDS) ordination based on Bray-Curtis distance matrices depicting the distribution patterns of bacterial and fungal communities in different plant compartments (root, stem, leave) and soil (rhizosphere and bulk soil). "Plant" represents the entire plant. "TC" represents the tissue-cultured plants, "WC" represents the plants under wild cultivation imitated method, and "WP" represents the wild plants.

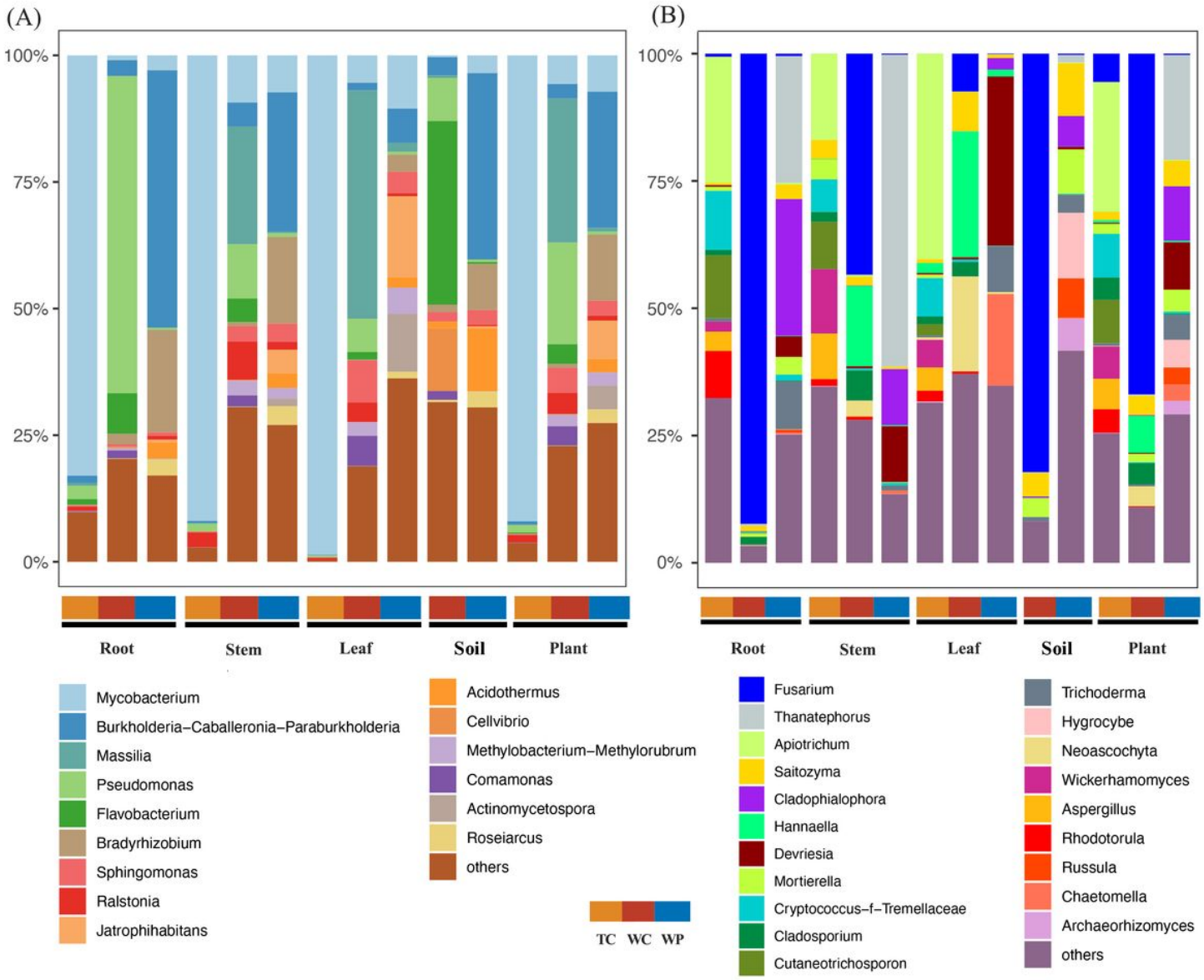


Figure 2

Taxonomic composition of bacterial and fungal microbiota. **(A)** The top 15 bacteria genus based on the relative abundance; (**B)** The top 20 fungi genus based on the relative abundance. The remaining genus were classified as "other".

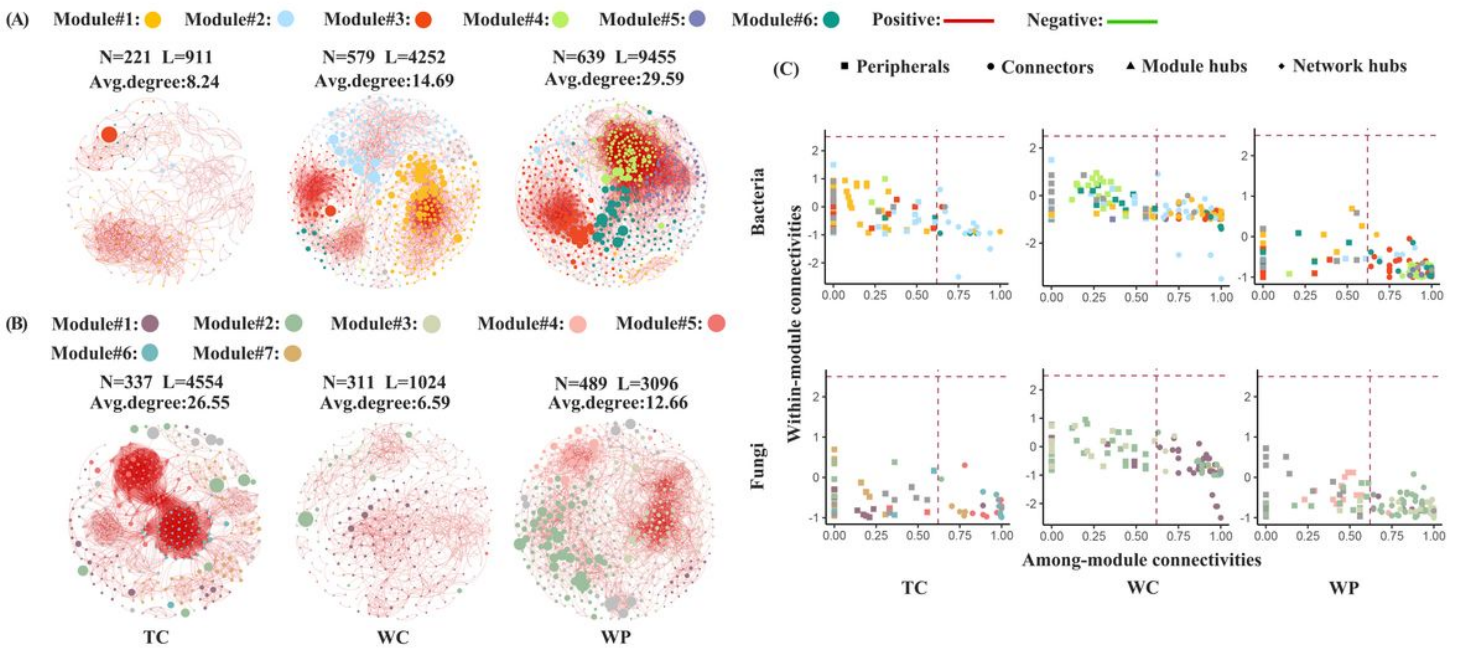


Figure 3

Co-occurrence network analysis showing microbial inter-kingdom network under different growth patterns. **(A)** Bacteria network. **(B)**Fungi network. **(C)** Keystone taxa and their modules in endophyte communities under three growth patterns. Module hubs (highly connected nodes within modules, Zi > 2.5), network hubs (highly connected nodes within the entire network, Zi > 2.5 and Pi > 0.62), and connectors (nodes that connect modules, Pi > 0.62)

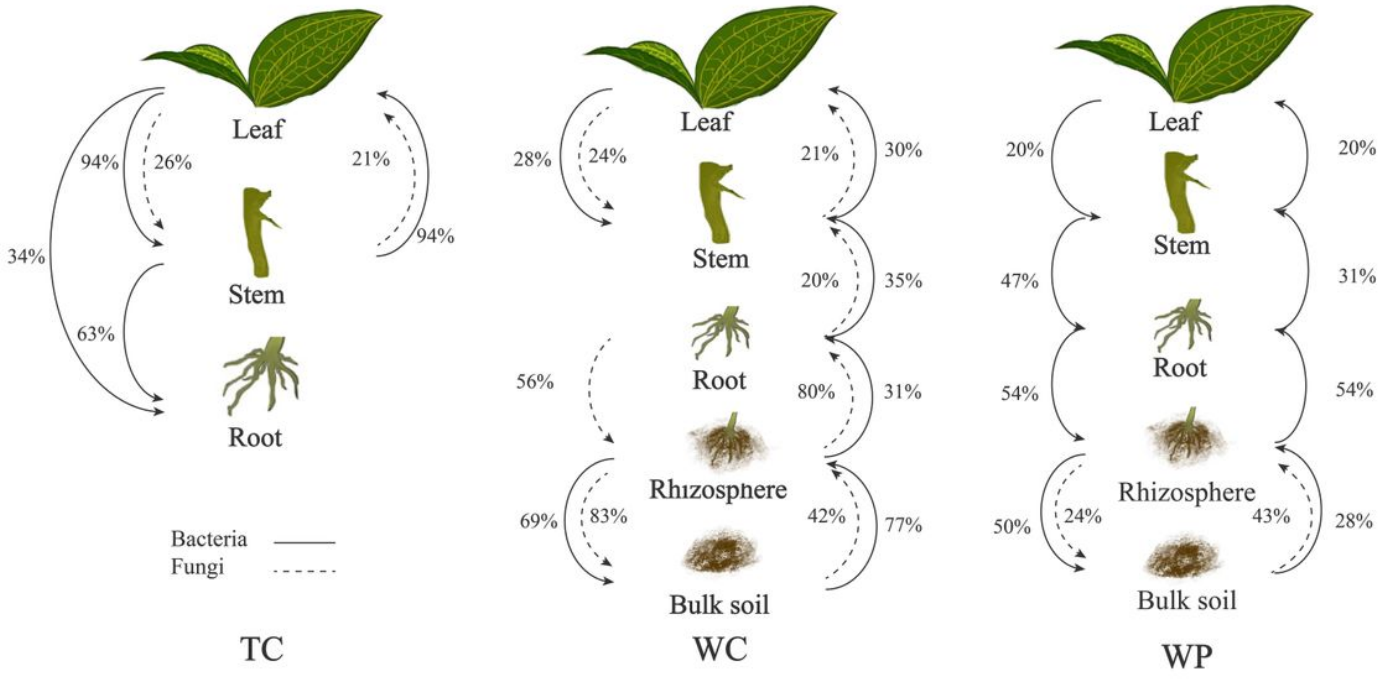


Figure 4

SourceTracker analysis results of *A. roxburghii* under different growth patterns.

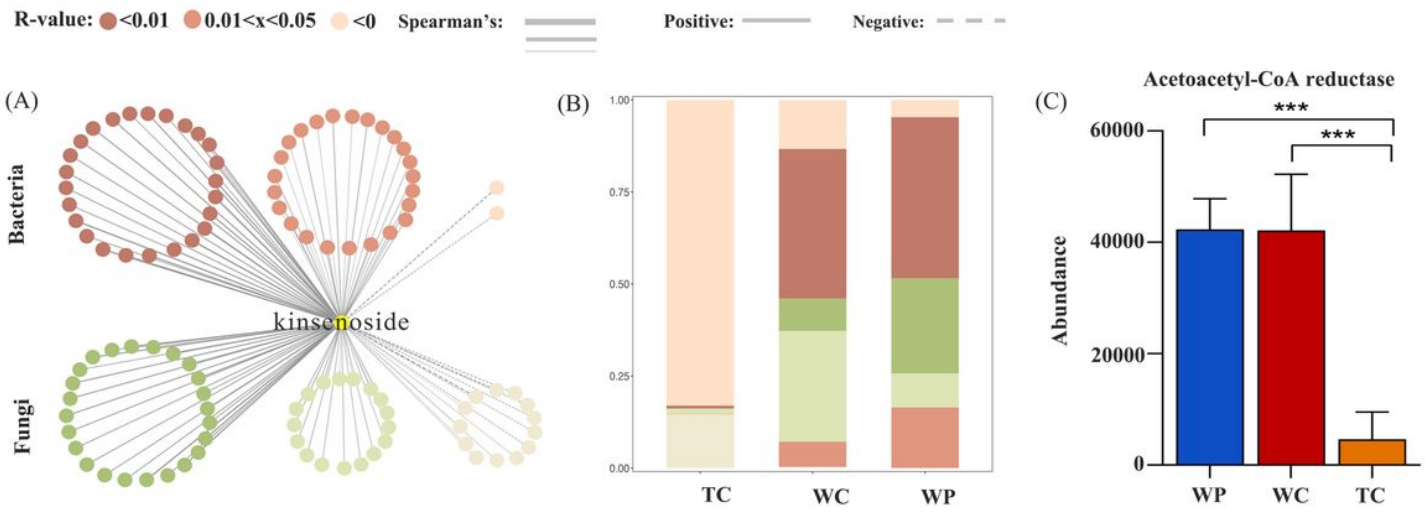


Figure 5

Correlation network and bar charts between core microbiota and the contents of kinsenoside. **(A)** Correlation network between core microbiota and the contents of kinsenoside. Endophytes with relative abundance greater than 100 were selected. **(B)** The composition of microbes which showing significant correlations with the contents of kinsenoside. Different colors represent sets of microbial communities with different correlations. (C)Relative abundance of Acetoacetyl-CoA reductase in different growth patterns

Supplementary Files

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