

The effect of rhizosphere soil on the flavonoid metabolism in the roots of *Tetrastigma hemsleyanum*

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Abstract

Background: *Tetrastigma hemsleyanum* (*T. hemsleyanum*) is a precious and rare traditional Chinese medicinal plant. Flavonoid is its main medicinal ingredient. Wild *T. hemsleyanum* (W-TH) growing in Zhejiang Province, China is recognized as a medicinal material of “San Ye Qing” Dao-di herbs. The different origins and thus the contents of medicinal ingredients are the key criteria used to determine whether the medicinal materials are authentic Dao-di herbs. However, it is less known how the eco-environments of its specific producing area, especially microbial community in rhizosphere soil, affect the content of medicinal ingredients in “San Ye Qing”. In the present study, we determined the content of total flavonoids and the enzymatic activity of phenylalanine ammonia-lyase (PAL) in the roots of W-TH and artificially cultivated *T. hemsleyanum* (C-TH), as well as the nutrient compositions and metagenome in rhizosphere soil. The effects of the rhizosphere soil on the flavonoid metabolism in *T. hemsleyanum* were evaluated.

Results: The contents of total flavonoids and the PAL activity were higher in W-TH root than in C-TH root. The contents of both available phosphorus and available potassium were higher in the rhizosphere soil of W-TH than in that of C-TH, while the contents of nitrogen and organic matter in the rhizosphere root of C-TH were higher than that of W-TH. Compared with the rhizosphere of C-TH, the abundances of genera *Enterobacter*, *Serratia*, *Raoultella*, *Kluyvera*, *Comamonas*, *Acinetobacter*, and *Arthrobacter*, and the pathways related to nitrogen metabolism, inositol phosphate metabolism, phosphinate metabolism, and phosphotransferase system in the rhizosphere of W-TH were significantly different. There existed differences in phenylpropanoid biosynthesis and phenylalanine metabolism in the rhizospheres of W-TH and C-TH.

Conclusions: The contents of nitrogen and available phosphorus in the rhizosphere soil affect diversity abundance, nitrogen metabolism, phosphorus metabolism, and phenylalanine (Phe) anabolism of rhizosphere microorganisms. They may further affect Phe content and PAL activity for the synthesis of flavonoids in the root of *T. hemsleyanum*.

Background

Tetrastigma hemsleyanum Diels & Gilg *ex* Diels (*T. hemsleyanum*) is a medical plant belonging to the family *Vitaceae* and the genus *Tetrastigma*. It is mainly distributed in the south of Yangtze River in China. It has strict demands for illumination and temperature in its habitat. Due to the excessive collection of the wild resources of *T. hemsleyanum* and the destruction of its suitable habitat, its population has been rapidly reduced. Thus, it has been listed as one of the endangered plants by both Zhejiang and Jiangxi Provinces in China [1–3].

T. hemsleyanum is rooted in Chinese medicine, and it, called “San Ye Qing” in Chinese, is one of the most important herbs in traditional Chinese medicine [4]. It has multiple medical values, including heat-clearing and toxin-detoxifying, phlegm-removing, blood circulation-promoting and pain-relieving, immunity-

regulating functions, as well as anti-inflammatory and anti-virus effects [5, 6]. Especially in recent years, *T. hemsleyanum* has been found to have anti-tumor effects [7–10]. Wild *T. hemsleyanum* (abbreviation as W-TH) growing in Zhejiang Province is recognized as a medicinal material of “San Ye Qing” Dao-di herbs [4], which has been a clinically preferred Chinese medicine for long time and is produced in a specific region with a specific production process. Dao-di herbs has higher quality and better curative effect compared with those of other medicinal materials [11, 12] and its formation is a complex evolution process, involving multiple factors. The different origins and thus the content of medicinal ingredients are the key criteria used for determining whether medicinal materials are authentic Dao-di herbs [13–15]. Yuan *et al.* (2015) reported that in addition to traditional genetics, epigenetics also plays an important role in formation of Dao-di herbs. They proposed epigenetic mechanism in the study of Dao-di herbs formation from specific phenotype and regional analysis [16]. Wang *et al.* (2018) deemed that Dao-di herbs had been recognized as “quality models” with a high status. The advancement of various omics technologies has provided new methods for the analysis of complex biological systems, which are also suitable for studying the quality formation in Dao-di herbs as well. With achievements of omics in the study of Dao-di herbs from the genetics to phenotyping, the use of these new methods of quality evaluation can be investigated in the biosynthetic pathways of secondary metabolites and the interaction with human body [17]. Hao *et al.* (2019) reported that as a treasure of traditional Chinese medicine, Dao-di herbs are famous for their high quality and good effect. However, traditional characterization of Dao-di herbs and their producing areas is mostly confined to qualitative description but lacks the objective evaluation indicators [18]. Specially, Fu *et al.* (2019) investigated the effects of different nitrogen levels on the growth of *T. hemsleyanum* and the content of phytochemicals and antioxidant activity in its stems and leaves. They found that a certain amount of nitrogen had positive effects on most of the biological traits but excessive dose of nitrogen went against growth of *T. hemsleyanum*. With the increase in nitrogen levels, the polysaccharide contents in stems and leaves were not significantly changed, while the contents of total flavonoids and phenolic components, and antioxidant activities were increased steadily. Antioxidant activities and contents of total flavonoid and phenolic components showed a significant and positive correlation [19].

W-TH growing in Zhejiang Province is the authentic Dao-di herbs of “San Ye Qing”. However, it is less known how the eco-environments in its specific producing area, especially microbial community in the rhizosphere soil affect the content of medicinal ingredients in “San Ye Qing”. In the present study, the metagenome in the rhizosphere soil of W-TH and the artificially cultivated *T. hemsleyanum* (abbreviation as C-TH) in greenhouse were determined. The effects of the microbial community in rhizosphere soil on the medicinal components of *T. hemsleyanum* were analyzed, aiming to provide the basis for further elucidating the mechanism underlying the formation of authentic Dao-di herbs.

Results

Flavonoid content, PAL activity in roots and nutrient content in rhizosphere soil

The measurements of flavonoid content, PAL activity and nutrient content showed that (1) the content of total flavonoids in W-TH roots was more than two times that of C-TH (Fig. 1), (2) the PAL activity of W-TH roots was higher than that of C-TH (Fig. 2) and (3) the contents of available phosphorus and available potassium in root soil of W-TH were higher than that of C-TH, while the contents of nitrogen and organic matter were higher in C-TH than in W-TH (Table 1). There were significant differences in root flavonoid content, PAL activity in root and contents of soil nutrient between W-TH and C-TH.

Sequencing results of rhizosphere soil metagenome, relative abundance and analysis of microbial organisms at genus level

The originally down-loaded data of sequencing and the assembled scafigs were provided in additional files 1 and 2: Tables S1 and S2. The sequencing results meet the requirements for analysis. For the assembled scafigs, the fragments with fewer than 500 bp were filtered out, and MetaGeneMark was used for Open Reading Frame (ORF) prediction, which was obtained for gene catalogue (Unigenes). The Unigenes were compared with the bacterial, fungal, archaea, and viral sequences extracted from the NCBI nr database to obtain species annotation information for W-TH and C-TH (additional files 3: Table S3). At the genus level, the abundances of the genera including *Burkholderia*, *Enterobacter*, *Bradyrhizobium*, *Paraburkholderia*, *Acinetobacter*, *Arthrobacter*, etc were determined. There were significant differences in community diversity distribution between W-TH and C-TH. A histogram showing the relative abundances of the top 10 genera were drawn (Fig. 3). Analysis of variance on W-TH vs. C-TH was performed using DEGseq software. The screening results were corrected using the Benjamini and Hochberg method (BH). Compared with those in C-TH, the genera *Enterobacter*, *Serratia*, *Raoultella*, *Kluyvera*, *Comamonas*, *Acinetobacter*, and *Arthrobacter* in W-TH were more abundant. The significantly up-regulated profiles in the top 10 genera were drawn as a bar graph (Fig. 4). The color is displayed according to the logFC size, and the volcano of the differential genera on W-TH vs. C-TH was shown in additional files 4: Fig. S1. According to the consistency of the genera shown in Figs. 2 and 3, genera *Enterobacter*, *Acinetobacter* and *Arthrobacter* were highly abundant and significantly up-regulated in W-TH.

Analysis of microbial metabolism pathways

Three levels are set for differential pathway levels: level 1 is the biological metabolic pathway, level 2 is the sub-function of the biological metabolic pathway, and level 3 is the detailed metabolic pathway of the sub-function. The compression Fisher test between W-TH and C-TH revealed that there were 3 significantly differential pathways at level 1 (additional files 5: Table S4), 19 significantly differential pathways at level 2 (additional files 6: Table S5), and 103 significantly differential pathways at level 3 (Table 2 and additional files 7: Table S6). The differential pathways in level 1 between W-TH vs C-TH are mainly related to genetic information processing and environmental information processing. A large number of sub-pathways related to metabolism pathways at level 2, including glycan biosynthesis and metabolism, folding, sorting and degradation, metabolism of cofactors and vitamins, nucleotide metabolism; amino acid metabolism; and lipid metabolism etc. Moreover, the specific metabolic

pathways with specific functions were further explored. At level 3, the significantly differential pathways included the following: inositol phosphate metabolism (phosphonate and phosphinate metabolism), tropane, piperidine and pyridine alkaloid biosynthesis, phenylpropanoid biosynthesis, nitrogen metabolism, phenylalanine metabolism, and phosphotransferase system (PTS), *etc.*

Discussion

Flavonoids are one group of the main medicinal ingredients of herbs “San Ye Qing”. Li *et al.* (2016) reported that there are significant differences in the contents of total flavonoids of 9 root samples of *T. hemsleyanum* collected from different producing areas in Guangxi Province [20]. Fan *et al.* (2013) also reported that there are significant differences in flavonoid contents in the root of *T. hemsleyanum* growing in Fujian and Zhejiang Provinces [21]. In this study, we observed significant differences in the flavonoid contents in the root of W-TH growing in Lishui, Zhejiang Province and C-TH in Hangzhou, Zhejiang Province. However, Ji *et al.* (2012) determined the content of flavonoids in the field of wild water cultivation and wild *T. hemsleyanum* in Lishui, Zhejiang Province. They found no significant difference between the two samples [22]. Wang *et al.* (2017) analyzed three different cultivated habitats of *T. hemsleyanum* in Zhejiang Province, and compared with the wild habitat under broad-leaved forest, the contents of total flavonoids cultivated under ridge culture and under bamboo forest were not significantly different [23]. In the studies conducted by Ji *et al.* (2012) and Wang *et al.* (2017) [22, 23], the soil used for artificially cultivation was derived from the same area, and is consistent with the soil of wild *T. hemsleyanum*, while the soil that we used for cultivation of *T. hemsleyanum* in greenhouse was the culture soil. Thus, the soil sources used for growth *T. hemsleyanum* are completely different, which may be the reason why our results are different from those reported by Ji *et al.* (2012) and Wang *et al.* (2017) [22, 23]. These results show that the soil conditions directly affect the flavonoid contents in the root of *T. hemsleyanum*.

In the study, we observed that the contents of nitrogen and organic matter were higher in C-TH root soil than in W-TH root soil, while the contents of available phosphorus and available potassium were higher in W-TH root soil than in C-TH root soil. Furthermore, the results of metagenomic analysis showed that the abundances of genera *Enterobacter*, *Serratia*, *Raoultella*, *Kluyvera*, *Comamonas*, *Acinetobacter*, and *Arthrobacter* in the root soil of W-TH were different from those in C-TH. The pathways related to nitrogen and phosphorus metabolism were also different, *i.e.* nitrogen metabolism, inositol phosphate metabolism, phosphonate and phosphinate metabolism, and phosphotransferase system (PTS) were different in the rhizospheres of W-TH and C-TH. These results suggest that the contents of nitrogen content and available phosphorus in the soil may change the diversity and abundance of the above-mentioned microorganisms, and the corresponding nitrogen and phosphorus metabolic pathways would be different, which will further affect the growth and development of *T. hemsleyanum*.

It is worth noting that comparing W-TH with C-TH, there exist differences in the bacterial genera and secondary metabolism, including tropane, piperidine and pyridine alkaloid biosynthesis, phenylpropanoid biosynthesis, phenylalanine metabolism, especially the phenylalanine metabolism. It is known that

phenylalanine (Phe) is the starting point of phytoflavonoid biosynthesis, and PAL is the first key enzyme of flavonoid synthesis. PAL catalyzes the formation of cinnamic acid and coumaric acid from Phe and is the key to the connection of phenylpropane compounds and primary metabolism. This enzyme plays an important role in regulating the biosynthesis of flavonoids [24]. It has been shown that the exogenous addition of appropriate amount of Phe can promote the plant to synthesize more secondary metabolite anthocyanins, and also restore the mutant phenotype with low contents of anthocyanin. Phe plays an important role in the secondary metabolism of anthocyanin synthesis [25]. The PAL activity in root of W-TH is higher than that in C-TH, while the “mother” plant of C-TH is derived from W-TH, which has the same genetic background of W-TH. Considering that the physiological features of C-TH and W-TH growth are similar, only the root soils for their growth are different, but the flavonoid content in W-TH is significantly higher than that in C-TH. This suggests that the contents of nitrogen and phosphorus in the soil affect the microbial abundance, the metabolisms of nitrogen and phosphorus, and the anabolism of phenylalanine. These factors further affect the Phe content and PAL activity in the root of *T. hemsleyanum*, which, in turn, affects the anabolism of flavonoids. However, how do the bacterial genera present in root soil affect the Phe content and PAL activity of *T. hemsleyanum* still needs to be explored. In addition, it is unclear whether soil pH and available potassium affect the anabolism of flavonoids in *T. hemsleyanum* and this aspect needs to be addressed as well.

Conclusions

In the present study, we determined the content of total flavonoids and the enzymatic activity of PAL in the roots of W-TH and C-TH growing in Zhejiang Province, China, as well as the nutrient composition and metagenome in rhizosphere soil. The effects of the rhizosphere soil on flavonoid metabolism in *T. hemsleyanum* were evaluated. The results showed that the contents of nitrogen and available phosphorus in rhizosphere soil affected diversity abundance, the nitrogen metabolism and phosphorus metabolism, and the phenylalanine anabolism of rhizosphere microorganisms. These factors may further affect Phe content and PAL activity for the synthesis of flavonoids in the root of *T. hemsleyanum*.

Methods

Preparation of experimental materials

Wild *T. hemsleyanum* (W-TH) was collected from Mountain areas in Lishui, Zhejiang Province and identified by Dr. Ji Qingyong of Lishui Academy of Agricultural Sciences. The permission was obtained to collect plants of wild *T. hemsleyanum*. Because of scarcity of the wild plants and laborious collection, we obtained only several wild plants, which have been used up for analysis but not been deposited in a publicly available herbarium in the study. The C-TH was originally derived from the cuttings of W-TH, and a large number of cultivated seedlings have been planted and growing in the greenhouse of Hangzhou Normal University, Hangzhou, Zhejiang Province, China. The cultivated soil was purchased from Zhejiang Gomei Gardening Co., Ltd (Zhejiang, China). The plant age of *T. hemsleyanum* was about 5 years.

Determination of total flavonoids in root of *T. hemsleyanum*

Referring to the method of Du *et al.* (2015) and Song *et al.* (2017) [2, 26], the gourd-shaped roots with a diameter of about 1.5 cm were selected, cut and dried at 70°C for 6 h to form powder. Powder (0.1 g) was weighted and put into a 5 mL centrifuge tube and 1.5 mL of 50% ethanol was added. After being extracted with a sonicator for 0.5 h and then centrifuged at 11000 r/min, 50% ethanol was added to 2.5 mL. 1.0 mL of the total flavonoid extract obtained was placed in a 25 mL volumetric flask, 50% ethanol was added to 9 mL, and 1 mL of 5% NaNO₂ solution was added. The mixed solution was shaken for 10 min, then 10% Al(NO₃)₃ was added. 1 mL of the solution was shaken and allowed standing for 10 min. Finally, 10 mL of 10% NaOH solution was added and diluted to 25 mL with 50% ethanol, shaken well, and allowed standing for 15 min. The sample solution was measured chromatographically. The absorbance at 500 nm (*OD*₅₀₀) was measured with an ultraviolet spectrophotometer. The content of total flavonoids in the sample was calculated according to a standard curve. Rutin (Sangon Biotechnology, Shanghai, China) was used as the standard for total flavonoids.

Determination of PAL activity in root of *T. hemsleyanum*

Root samples (1.0 g) were weighted and 2.5 mL of PAL lysis buffer (Shanghai Xin Fan Biotechnology Co., Ltd.) was added. The mixture was fully mashed, ground into a homogenate in an ice bath, and finally centrifuged at 12000 r/min for 15 min at 4°C. Supernatant was cryopreserved at –20°C for measurement of PAL activity. The enzymatic activity was measured using a PAL ELISA Kit (Shanghai Xin Fan Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instruction. Under the experimental conditions, the amount of enzyme that changes the *OD*₂₉₀ value by 0.01 per hour is defined as one unit (U) of enzymatic activity.

Determination of nutrients in rhizosphere soil

The pH, the contents of available potassium, available phosphorus, total nitrogen and organic matter in rhizosphere soil of W-TH and C-TH were measured according to the Chinese Agricultural Standards NY/T1121.2–2006, NY/T889–2004, NY/T1121.7–2014 and NY/T53–1987 (<http://www.chinanyrule.com/tsLibIndex.html>). The instruments including METTLER TOLEDO FE28 portable acidity meter, Shimadzu AA–6880 series atomic absorption spectrophotometer and V–1000 visible spectrophotometer (Shanghai Precision Instrument Co., Ltd., Shanghai, China) were used.

Construction and sequencing of metagenomic library in rhizosphere soil

The genomic DNA was extracted from soil samples using the MoBio PowerSoil™ DNA Isolation Kit according to the method of Simmons *et al.* (2018) [27]. Purity and integrity of the extracted DNA samples were analyzed by 1% agarose gel electrophoresis. DNA concentration and purity were measured accurately at OD_{260} and OD_{260}/OD_{280} ratio with Nanodrop Qubit® 2.0 Fluorometer (Thermo Scientific, Waltham, USA). The qualified DNA samples were randomly fragmented into pieces of about 300 bp in length using a Covaris sonicator and used for library construction. Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA), following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq2500 platform (250 bp paired-end reads).

Metagenome analysis

The raw data obtained by sequencing were filtered to obtain clean data, which were then assembled with scaftigs [28–30]. Gene prediction was performed using MetaGeneMark [31, 32] to construct gene catalogue (Unigenes). From the gene catalogue, the clean data of each sample were combined to obtain the information about abundance of the gene catalogue in each sample. Unigenes was compared to microNR database in NCBI using DIAMOND software [33], and species annotation information for this sequence was further determined using the LCA algorithm [34]. From the LCA annotated results and the gene abundance table, the abundance information of each sample at each classification level (*e.g.* genus and species) was obtained. Abundance histogram display, principal component analysis (PCA) and significant difference analysis were performed. To determine whether there was a significant difference in the genus level between the samples, DEGseq [35] was used for differential analysis, and corrected by Benjamini and Hochberg method (BH) ($q \leq 0.001$, $\log FC \geq 1$), with a focus on up-regulated bacterial group (at genus level). From the gene catalogue, KEGG, eggNOG and CAZy analysis were performed [36–38], and a Fisher test ($p < 0.05$) between samples for differential functional abundance was performed. Cluster analysis and metabolic pathway analysis were also performed.

Statistical analysis

The total flavonoids in root of *T. hemsleyanum* and the nutrient composition in root soil were measured three times, and statistical analysis was performed using SPSS software (version 19.0). The difference between groups with $p < 0.05$ was regarded statistically significant. The experimental data were expressed as the mean value $\pm$ standard deviation (SD) of the results of repeated experiments. Three rhizosphere

soil samples of W-TH and C-TH were collected respectively. After being mixed well, the soil samples were used to determine the respective metagenomic groups of W-TH and C-TH.

Abbreviations

CAZy: Carbohydrate-Active Enzymes; C-TH: cultivated *Tetrastigma Hemsleyanum*; eggNOG: evolutionary genealogy of genes: Non-supervised Orthologous Groups; ELISA: Enzyme Linked Immunosorbent Assay; KEGG: Kyoto Encyclopedia of Genes and Genomes; LCA: Least Common Ancestors; NCBI: National Center for Biotechnology Information; OD:Optical Density; ORF: Open Reading Frame; PAL: Phenylalanine Ammonia-lyase; PCA: Principal Component Analysis; W-TH: wild *Tetrastigma Hemsleyanum*

Declarations

Ethics approval and consent to participate

The collection of wild *T. Hemsleyanum* has been permitted for scientific research purposes by Lishui Forestry Administrative Organization. Field planting was conducted in accordance with local legislation. This study adhered to all relevant institutional, national and international guidelines.

Consent for publication

Not applicable.

Availability of data and material

All the data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TX designed the experiments, performed the data analysis, and drafted the manuscript. XT helped in growing the plants and sample collection. JL, SB, ZX and XZ carried out analysis of PAL activity and flavonoids contents. CL helped in performing data analysis and growing the plants. All the authors read and approved the final text.

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Tables

Table 1

	W-TH	C-TH
pH	5.68±0.09	4.72±0.09
Nitrogen (%)	0.088±0.04	1.057±0.05
Organic matter (g/Kg)	33.2±0.5	284±3.8
Available phosphorus (mg/Kg)	43.4±4.0	15.7±1.3
Available potassium (mg/Kg)	409±10.0	198±5.4

Table 2

Metabolic pathway	<i>p</i> value	interval lower	interval upper	<i>q</i> value
Inositol phosphate metabolism	1.81E-09	1.2259	1.4987	1.64E-08
Phosphonate and phosphinate metabolism	1.56E-05	1.2340	1.7702	6.42E-05
Biosynthesis of unsaturated fatty acids	9.48E-04	1.0641	1.2789	3.12E-03
Citrate cycle (TCA cycle)	1.58E-04	0.8526	0.9512	5.89E-04
Pyrimidine metabolism	9.53E-18	0.7929	0.8649	2.01E-16
Xylene degradation	2.00E-16	1.7795	2.6065	3.90E-15
Metabolism of xenobiotics by cytochrome P450	1.55E-12	1.8055	2.9486	1.87E-11
Starch and sucrose metabolism	2.82E-05	0.8511	0.9436	1.12E-04
Steroid degradation	1.14E-05	1.2627	1.8696	4.97E-05
One carbon pool by folate	3.74E-06	0.7503	0.8916	1.82E-05
Bisphenol degradation	4.14E-18	1.2826	1.4861	1.05E-16
Tyrosine metabolism	1.57E-05	1.0796	1.2279	6.42E-05
Glycerolipid metabolism	1.45E-02	1.0286	1.3107	3.75E-02
Tropane, piperidine and pyridine alkaloid biosynthesis	4.59E-07	1.1969	1.5102	2.64E-06
Chlorocyclohexane and chlorobenzene degradation	3.03E-08	1.1996	1.4697	2.07E-07
Ubiquitin mediated proteolysis	1.14E-08	0.0685	0.3501	9.03E-08

Figures

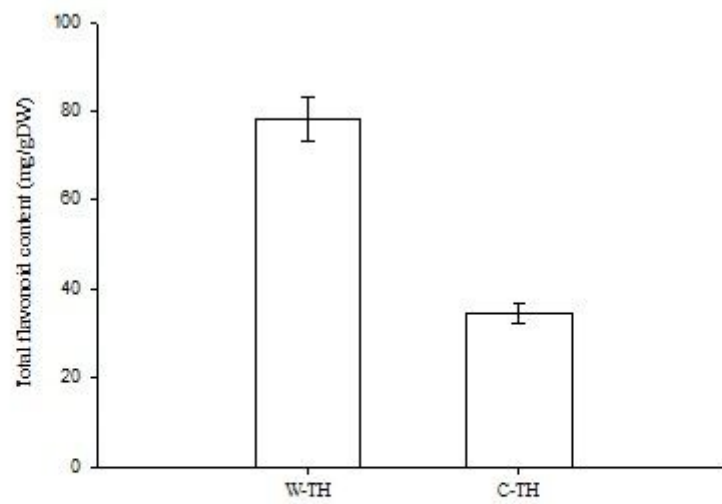


Fig. 1

Figure 1

Contents of total flavonoids in roots of W-TH and C-TH.

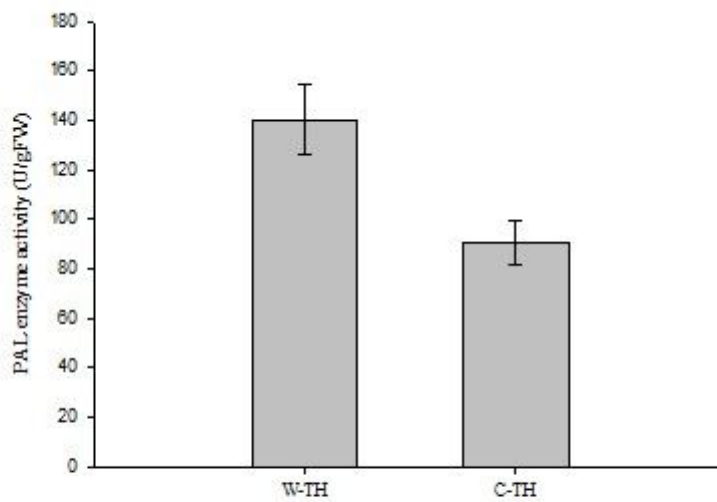


Fig. 2

Figure 2

PAL activities in roots of W-TH and C-TH.

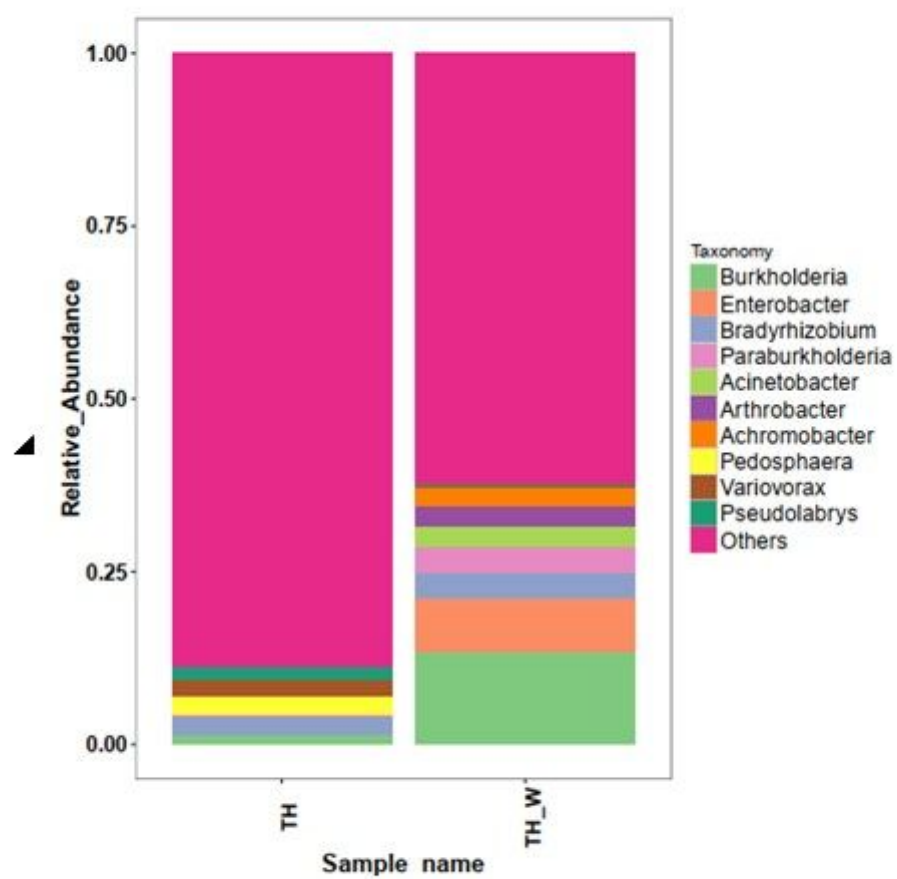


Fig. 3

Figure 3

Relative abundance distribution profiles of the top 10 genera between W-TH and C-TH.

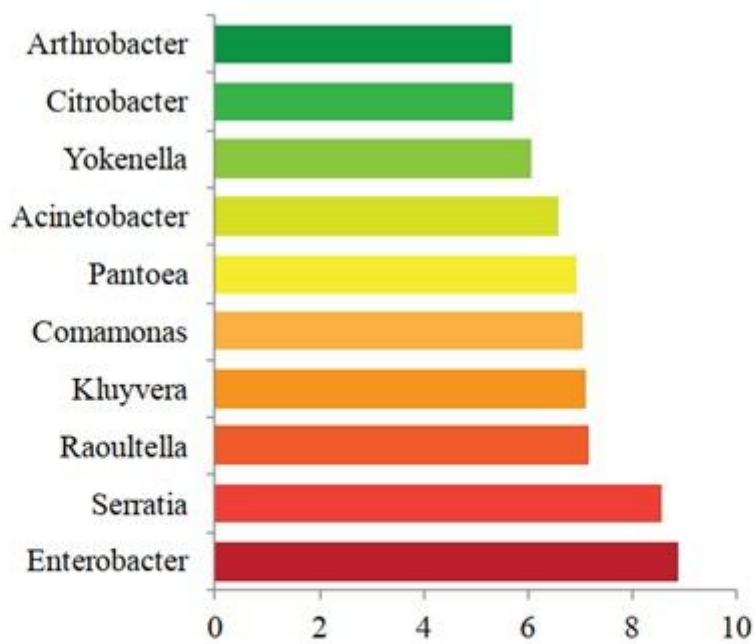


Fig. 4

Figure 4

The significantly up-regulated profiles of the top 10 genera by W-TH vs. C-TH.

Supplementary Files

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