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EFFECT OF RHIZOSPHERE SOIL ON FLAVONOID METABOLISM IN ROOTS OF *Tetrastigma hemsleyanum*

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Tetrastigma 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. 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 P and available K were higher in the rhizosphere soil of W-TH than in that of C-TH, while the contents of N 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*, *Acinetobacte*, and *Arthrobacter*, and the pathways related to N 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. The contents of N and available P in the rhizosphere soil affect diversity abundance, N metabolism, Pmetabolism, 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*.

Keywords: *Tetrastigma hemsleyanum* Diels & Gilg*ex* Diels, Rhizosphere soil, Root, Microbial community, Flavonoid, Dao-di herbs.

INTRODUCTION

Tetrastigma hemsleyanum Diels & Gilg*ex* Dielsis 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 light 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 (Qian *et al.*, 2015; Du *et al.*, 2015; Yin *et al.*, 2018).

Tetrastigma 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 (Zhu and Yan, 2014). 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 (Sun *et al.*, 2013; Wang *et al.*, 2018). Recent research has revealed that *T. hemsleyanum* has anti-tumor effects (Feng *et al.*, 2014; Xiong *et al.*, 2015; Peng *et al.*, 2016; Chen *et al.*, 2018). Wild *T. hemsleyanum* (abbreviation as W-TH) growing in Zhejiang Province is recognized as a medicinal material of "San Ye Qing" Dao-di herbs (Zhu and Yan, 2014), 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 (Han *et al.*, 2011; Zhao *et al.*, 2012) 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 (Wang, 2011; Zhang *et al.*, 2016; Yuan *et al.*, 2017). 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 (Yuan *et al.*, 2015). 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. However, traditional characterization of Dao-di herbs and their producing areas is mostly confined to qualitative description but lacks the objective evaluation indicators (Hao *et al*., 2019). 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 N had positive effects on most of the biological traits, but excessive dose of N went against growth of *T. hemsleyanum*. With the increase in N 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.

W-TH growing in Zhejiang Province is the authentic Dao-di herbs of "San Ye Qing". However, it is less known how the eco-environment in its specific producing area, especially microbial community in the rhizosphere soil affects 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.

MATERIALS AND 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 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), 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% $NaNO_2$ solution was added. The mixed solution was shaken for 10 min, then 10% $Al(NO_3)_3$ 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 K, available P, total N 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 MoBioPowerSoil™ DNA Isolation Kit according to the method of Simmons *et al.* (2018). 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 Covarissonicator 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 (Mende *et al.*, 2012; Karlsson *et al.*, 2013; Nielsen *et al.*, 2014). Gene prediction was performed using MetaGeneMark (Zhu et al, 2010; Li et al, 2014) 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 (Buchfink *et al.*, 2015), and species annotation information for this sequence was further determined using the LCA algorithm (Hanson *et al.*, 2016). 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 (Wang *et al.*, 2009) was used for differential analysis, and corrected by Benjamini and Hochberg method (BH) ($q \leq 0.001$, logFC≥ 1), with a focus on up-regulated bacterial group (at genus level). From the gene catalogue, KEGG, eggNOG and CAZy analysis were performed (Levasseur *et al.*, 2013; Kanehisa *et al.*, 2016; Huerta-Cepas *et al.*, 2016), 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. After being mixed well, the soil samples were used to determine the respective metagenomic groups of W-TH and C-TH.

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) and (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 N 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.

Table 1. Results of nutrients in rhizosphere soils of W-TH and C-TH.

	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

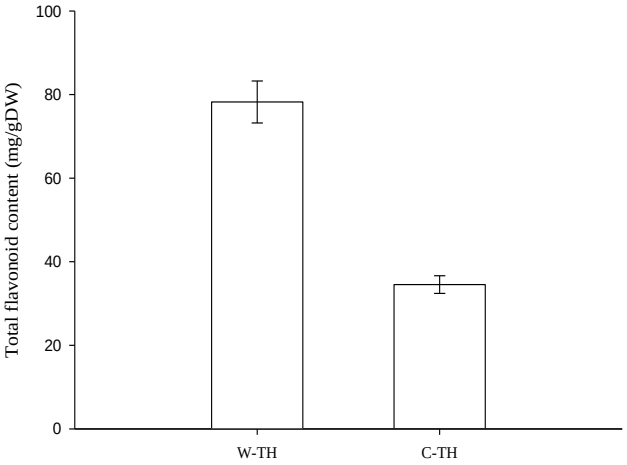

Figure 1. Contents of total flavonoids in roots of W-TH and C-TH.

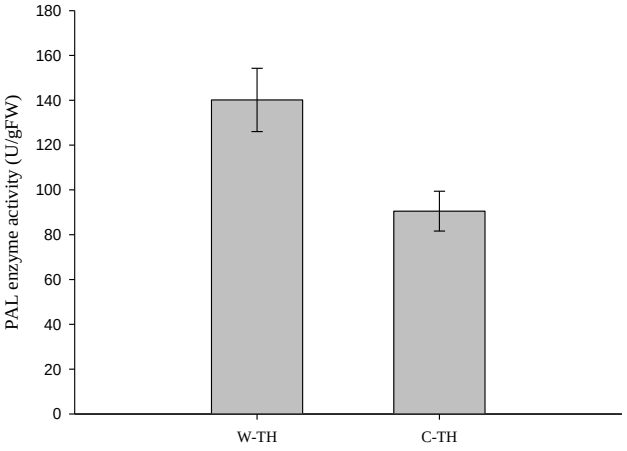

Figure 2. PAL activities in roots of W-TH and C-TH.

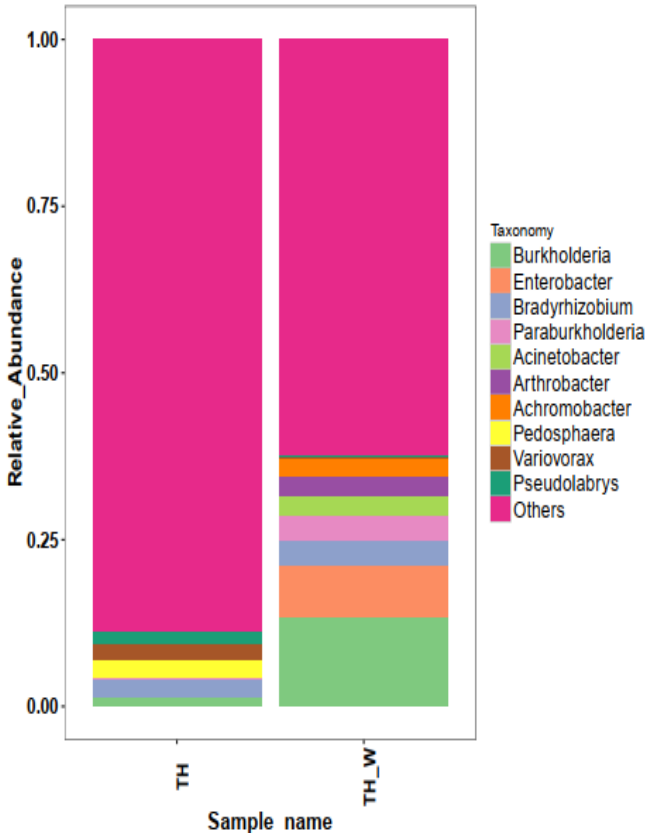

Figure 3. Relative abundance distribution profiles of the top 10 genera between W-TH and C-TH.

Sequencing results of rhizosphere soil metagenome, relative abundance and analysis of microbial organisms at genus level: 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. 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 (Figure 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*, *Acinetobacte*, 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). According to the consistency of the genera shown in Figures 2 and 3, genera *Enterobacter*, *Acinetobacter* and *Arthrobacter* were highly abundant and significantly up-regulated in W-TH.

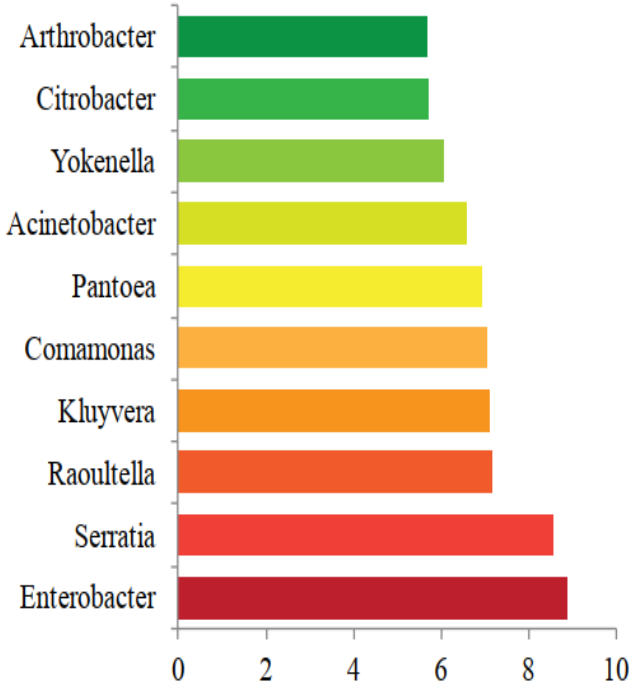

Figure 4. The significantly up-regulated profiles of the top 10 genera by W-TH *vs*. C-TH.

Analysis of microbial metabolism pathways: Three levels were set for differential pathway levels: level 1 was the biological metabolic pathway, level 2 was the sub-function of the biological metabolic pathway, and level 3 was 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, 19 significantly differential pathways at level 2, and 103 significantly differential pathways at level 3 (Table 2). The differential pathways in level 1 between W-TH *vs* C-TH were mainly related to genetic information processing and environmental information processing. A large number of sub-pathways related to metabolism pathways at level 2, included 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*.

Table 2. Differential metabolic pathways of the top 16 by W-TH *vs.* C-TH.

Metabolic pathway	*p* value	interval lower	interval upper	*q* 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

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. 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. 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. 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. In the studies conducted by Ji *et al.* (2012) and Wang *et al.* (2017), 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). These results show that the soil conditions directly affect the flavonoid contents in the root of *T. hemsleyanum*.

In the study, it was observed that the contents of N and organic matter were higher in C-TH root soil than in W-TH root soil, while the contents of available P and available K 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*, *Acinetobacte*, *and Arthrobacter* in the root soil of W-TH were different from those in C-TH. The pathways related to N and P metabolism were also different, *i.e*. N 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 N content and available P in the soil may change the diversity and abundance of the above-mentioned microorganisms, and the corresponding N and P 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 phyto flavonoid 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 (Jones, 1984). 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 (Chen *et al.*, 2016). The PAL activity in root of W-TH was higher than that in C-TH, while the "mother" plant of C-TH was derived from W-TH, which had the same genetic background of W-TH. Considering that the

physiological features of C-TH and W-TH growth were similar, only the root soils for their growth were different, but the flavonoid content in W-TH was significantly higher than that in C-TH. This suggests that the contents of N and P in the soil affect the microbial abundance, the metabolisms of N and P, and the anabolism of phenylalanine. These factors further affected the Phe content and PAL activity in the root of *T. hemsleyanum*, which, in turn, affected the anabolism of flavonoids. However, how did the bacterial genera present in root soil affected the Phe content and PAL activity of *T. hemsleyanum* still needs to be explored. In addition, it was unclear whether soil pH and available K affected 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-THgrowing 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 N and available P in rhizosphere soil affected diversity abundance, the N metabolism and Pmetabolism, 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*.

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REFERENCES

Buchfink, B., C. Xie and D. H. Huson.2015. Fast and sensitive protein alignment using DIAMOND. Nat. Methods. 12:59-60.

Chen, Q., C. Man, D. Li, H. Tan, Y. Xie and J. Huang. 2016. Arogenate dehydratase isoforms differentially regulate anthocyanin biosynthesis in *Arabidopsis thaliana*. Mol. Plant9:1609-1619.

Chen, S., M. Luo, L. Ma and W. Lin. 2018. Ethylacetate extract from *Tetrastigma hemsleyanum* inhibits proliferation and induces apoptosis in HepG2 and SMMC-7721 cells. Cancer Manag. Res. 10: 3793-3799.

Du, S., T. Xiang, Y. Song, L. Huang, Y. Sun and Y. Han.2015. Transgenic hairy roots of *Tetrastigma hemsleyanum*: induction, propagation, genetic characteristics and medicinal components. Plant Cell Tiss. Org. Cult. 122: 373-382.

Fan, S., Q. Lin, W. Xu, Y. Lin, Z. Bu and D. Chen.2013. Comparison of total flavonoids contents in *Tetrastigma hemsleyanum* from different regions. J. Fujian U. Tradit. Chin. Med. 23: 44-45).

Feng, Z., W. Hao, X. Lin, D. Fan and J. Zhou.2014. Antitumor activity of total flavonoids from *Tetrastigma hemsleyanum* Diels *et*Gilg is associated with the inhibition of regulatory T cells in mice. Onco. Targets Ther. 7: 947-756.

Fu, L., L. Zhao, H. Yu, M. Yan, Y. Zheng, Q. Liu, L. Jin, J. Cheng, T. Lu and L. Wang.2019. Effects of nitrogen level on growth of *Tetrastigma hemsleyanum* and phytochemical content and antioxidant activity in stems and leaves. Zhongguo Zhong Yao Za Zhi. 44: 696-702.

Han, B., H. Peng and L. Huang.2011. Research advances of Dao-di Herbs in China. Chin. J. Nature33: 281-285.

Hanson, N.W., K.M. Konwar and S. J. Hallam. 2016. LCA*: an entropy-based measure for taxonomic assignment within assembled metagenomes. Bioinformatics 32: 3535-3542.

Hao, D., L. Huang, Y. Yuan and Y. Jin.2019. The application of numerical taxonomy in the study of Dao-di herbs and their producing areas.Zhongguo Zhong Yao Za Zhi: on line first. doi: 10.19540/j.cnki.cjcmm.20190701.101.

Huerta-Cepas, J., D. Szklarczyk, K. Forslund, H. Cook, D. Heller, M. C. Walter, T. Rattei, D.R. Mende, S. Sunagawa, M. Kuhn, L.J. Jensen, C. von Mering and P. Bork.2016. eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. Nucleic Acids Res. 44: D286-D293.

Ji, Q., J. Hua, W. Chen, B. Zhu, X. Wang and M. Li.2012. Comparison total flavonoids contents between Cultivated and wild-type *Tetrastigma hemsleyanum*. J. Zhejiang Agric. Sci. VOL: 37-38.

Jones, D.H. 1984. Phenylalanine ammonia-lyase: regulation of its induction, and its role in plant development. Phytochem. 23:1349-1359.

Kanehisa, M., Y. Sato, M. Kawashima, M. Furumichi and M. Tanabe.2016. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 44: D457-D462.

Karlsson, F.H., V. Tremaroli, I. Nookaew, G. Bergstrom, C.J. Behre, B. Fagerberg, J. Nielsen and F. Backhed. 2013. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature 498: 99-103.

Levasseur, A., E. Drula, V. Lombard, P.M. Coutinho and B. Henrissat. 2013. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. Biotechnol. Biofuels. 6:41, doi: 10.1186/1754-6834-6-41.

Li, J., H. Jia, X. Cai, H. Zhong, Q. Feng, S. Sunagawa, M. Arumugam, J.R. Kultima, E. Prifti, T. Nielsen, A.S. Juncker, C. Manichanh, B. Chen, W. Zhang, F. Levenez,

J. Wang, X. Xu, L. Xiao, S. Liang, D. Zhang, Z. Zhang, W. Chen, H. Zhao, J.Y. Al-Aama, S. Edris, H. Yang, J. Wang, T. Hansen, H.B. Nielsen, S. Brunak, K. Kristiansen, F. Guarner, O. Pedersen, J. Doré, S.D. Ehrlich, M. Consortium, P. Bork and J. Wang.2014. An integrated catalog of reference genes in the human gut microbiome. Nature biotech. 32: 834-841.

Mende, D.R., A.S. Waller, S. Sunagawa, A.I. Järvelin, M.M. Chan, M. Arumugam, J. Raes and P. Bork.2012. Assessment of metagenomic assembly using simulated next generation sequencing data.PloS one 7: e31386.

Nielsen, H.B., M. Almeida, A.S. Juncker, S. Rasmussen, J. Li, S. Sunagawa, D.R. Plichta, L. Gautier, A.G. Pedersen, E.L. Chatelier, E. Pelletier, I. Bonde, T. Nielsen, C. Manichanh, M. Arumugam, J. Batto, N. Blom, N. Borruel, K.S. Burgdorf, F. Boumezbeur, F. Casellas, J. Doré, P. Dworzynski, F. Guarner, T. Hansen, F. Hildebrand, R. Kaas, S. Kennedy, K. Kristiansen, J.R. Kultima, P. Léonard, F. Levenez, O. Lund, B. Moumen, D.L. Paslier, N. Pons, O. Pedersen, E. Prifti, J. Qin, J. Raes, S. Sørensen, J. Tap, S. Tims, D.W. Ussery, T. Yamada, M. Consortium, P. Renault, T. Sicheritz-Ponten, P. Bork, J. Wang, S. Brunak and S.D. Ehrlich. 2014. Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. Nature biotech. 32:822-828.

Peng, X., Y.Y. Zhang, J. Wang and Q. Ji.2016. Ethylacetate extract from *Tetrastigma hemsleyanum* induces apoptosis via the mitochondrial caspase-dependent intrinsic pathway in HepG2 cells. Tumour Biol. 37:865-876.

Qian, L., D. Dai, H. Jiang and W. Lin.2015. Research progresses of the endangered medicinal plant *Tetrastigma hemsleyanum* Diels *et*Gilg. Acta Agri. Zhejiang. 27:1301-1308.

Simmons, T., D.F. Caddell, S. Deng and D. Coleman-Derr. 2018. Exploring the root microbiome: extracting bacterial community data from the soil, rhizosphere, and root endosphere. J. Vis. Exp. (135): e57561, doi: 10.3791/57561.

Song, Y., P. Wu, Y. Li, X. Tong, Y. Zheng, Z. Chen, L. Wang and T. Xiang.2017. Effect of endophytic fungi on the host plant growth, expression of expansin gene and flavonoid content in *Tetrastigma hemsleyanum* Diels Gilg*ex* Diels. Plant Soil 417: 393-402.

Sun, Y., H. Li, J. Hu, J. Li, Y.W. Fan, X.R. Liu and Z.Y. Deng.2013. Qualitative and quantitative analysis of phenolics in *Tetrastigma hemsleyanum* and their antioxidant and antiproliferative activities. J. Agric. Food Chem. 61:10507-10515.

Wang, C.Y., H.J. Jang, Y.K. Han, X.D. Su, S.W. Lee, M.C. Rho, H.S. Wang, S.Y. Yang and Y.H. Kim.2018. Alkaloids from *Tetrastigma hemsleyanum* and their anti-inflammatory effects on LPS-induced RAW264.7 cells. Molecules. 23: pii: E1445.

Wang, C.C., H. Zhao, Y. Yan, J.J. Shi, S.Y. Chen, M.X. Tan, J.L. Chen, Z.X. Liu, C.H. Chen, L.S. Zou and X.H. Liu. 2018. Thoughts of omics research on quality formation in Dao-di herbs. Zhongguo Zhong Yao Za Zhi.43:2407-2412.

Wang, L., Z. Feng, X. Wang, X. Wang and X. Zhang.2009. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics 26:136-138.

Wang, Y. 2011. Study on quality characteristics and the causes of Dao-di herb.*Zhongguo Zhong Yao Za Zhi.* 36: 1

Wang, S., J. Xu, L. Zhang, Y. Xu and G. Huang.2017. Effects of different cultivation habitats on growth and contents of trace elements and total flavonoids of *Tetrastigma hemsleyanum.* J. Zhejiang Agric. Sci. 58: 2177-2178.

Xiong, Y., X. Wu and L. Rao.2015. *Tetrastigma hemsleyanum* (Sanyeqing) root calabash-shaped root extracts induces apoptosis in human cervical carcinoma HeLa cells. J. Ethnopharmacol 65:46-53.

Yin, M., W. Xu, N. Xie, X. Zhan, Y. Liao, Z. Wan, H. Cai and R. Chen.2018. Genetic diversity analysis of *Tetrastigma hemsleyanum* germplasm resources based on fluorescently labeled SSR markers. Chin. Tradit. Herbal Drugs. 49:5649-5656.

Yuan, Y., T.Y. Chen, L.Q. Huang, Y. Jin, J. Yang and Y.Y. Zhao.2017. Research strategies and application of reference Dao-di herbs. Zhongguo Zhong Yao Za Zhi42:2623-2626.

Yuan, Y., Y. Wei, J. Yu and L.Q. Huang.2015. Relationship of epigenetic and Dao-di herbs. Zhongguo Zhong Yao Za Zhi 40: 2679-2683.

Zhang, X., Y. Zhao, X. Huang, Z. Qiu, L. Guo, X. Qu and L. Huang.2016. Review on study of Dao-di herbs *Artemisiae AnnuaeHerba*. Zhongguo Zhong Yao Za Zhi. 41:2015-2018.

Zhao, Z.Z., P. Guo and E. Brand.2012. The formation of daodi medicinal materials. J. Ethnopharmacol. 140:476-481.

Zhu, M.X. and L.H. Yan. 2014. Resource investigation on rare and endangered She medicine *Tetrastigma hemsleyanum* in Zhejiang Province. Zhong Yao Cai.37:766-770.

Zhu, W., A. Lomsadze and M. Borodovsky.2010. Ab initio gene identification in metagenomic sequences. Nucleic Acids Res. 38: e132-e132.

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