The Medicinal Plant *Atractylodes Lancea* Recruits Specialized Plant Growth-Promoting Bacteria From The Soil

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Research Article

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Abstract

Purpose: *Atractylodes lancea* is a medicinal plant used to treat rheumatic diseases, digestive disorders, night blindness, and influenza. However, the microbiome associated with *A. lancea* remains unclear. In this study, we assess the role of microorganisms in the roots of *A. lancea* in regulating plant growth and secondary metabolites, and investigate the microbial composition of the root of *A. lancea*.

Methods: The roots of *A. lancea* were inoculated with 10% soil suspension at different temperatures. Thereafter, the biological indices, major volatile oils, chemical properties of the rhizosphere soil, and the diversity of root endophytic and rhizosphere bacterial communities of *A. lancea* were assessed.

Results: Soil microorganisms could attenuate the damage of high-temperature to *A. lancea* and significantly promote the growth and accumulation of volatile oil. *A. lancea* recruited endogenous plant growth-promoting bacteria (PGPBs) from soil, including *Burkholderia-Caballeronia-paraburkholderia*, *Bradyrhizobium*, *Paenibacillus*, *Bacillus* and *Rhodococcus*. These bacteria were positively correlated with four volatile oils. In the rhizosphere, PGPBs such as Novosphingobium are recruited.

Conclusions: Soil microorganisms promote the growth and development of *A. lancea*, improve the plant's ability to resist high temperature stress, and accelerate secondary metabolite accumulation. Most importantly, *A. lancea* could recruit and enrich specialized PGPBs from the soil. The PGPBs were significantly and positively correlated with *A. lancea* secondary metabolite and soil nutrient content, and can be used as ideal biological material in *A. lancea* cultivation and quality improvement.

Introduction

In the natural ecosystem, soil microorganisms play a pivotal role in the geochemical cycle and the survival and development of plants (Schimel and Bennett 2004; Jacoby et al. 2017; Johns 2017). These microorganisms exhibit complex interactions and functions in plants (Zilber-Rosenberg and Rosenberg 2008; Vandenkoornhuyse et al. 2015), and are classified as beneficial, pathogenic, or neutral based on their interactions with the hosts (Berg et al. 2020). Plants recruit specific root microbiota from the soil by screening of root exudates and surfaces with abundant bacteria as well as their microbial immune system (Bulgarelli et al. 2013; Mendes et al. 2014; Reinhold-Hurek et al. 2015; Berg and Raaijmakers 2018; Zhong et al. 2019). Therefore, different plants correspond to various beneficial microbial compositions (Bergsma-Vlami et al. 2005; Aira et al. 2010; Ramachandran et al. 2011). Plant growth-promoting bacteria (PGPBs) promote plant growth by colonizing the rhizosphere or endophytic environment (Sousa and Olivares 2016). Rhizobia, including *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, and *Sinorhizobium*, are the most common PGPBs and can successfully establish effective symbiotic relationships with legumes (Hayat et al. 2010). Moreover, other PGPBs, such as *Frankia*, *Bacillus*, and *Pseudomonas*, can protect plants from diseases and abiotic stresses via various mechanisms, such as nitrogen fixation, mineral absorption promotion, niche competition, and production of plant hormones and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Tian et al. 2020). In

addition, studies have demonstrated that PGPBs can not only promote the growth of medicinal plants but also increase the accumulation of effective medicinal ingredients. The endophytic actinomycete *Pseudonocardia* sp. in *Artemisia annua* L. can upregulate the expression of enzyme genes related to the artemisinin biosynthesis pathway, thereby promoting the accumulation of artemisinin (Li et al. 2012). Similarly, inoculation of endophytic bacteria, *Staphylococcus sciuri* and *Micrococcus* sp., can increase the contents of vindoline, serpentine, and ajmalicine in *Catharanthus roseus* (Tiwari et al. 2013). Therefore, the application of PGPBs as a bioinoculant is a promising and sustainable strategy for enhancing medicinal plant yield and quality improvement.

Atractylodes lancea is an important medicinal plant with remarkable medicinal and economic values; the volatile oils of this plant are used as main active pharmaceutical ingredients in the treatment of rheumatic diseases, digestive disorders, night blindness, and influenza (Wang et al. 2008; Nie 2018; Qu et al. 2018). The cultivation area of *A. lancea* is expanding every year; however, traditional cultivation methods are not conducive to sustainable development of the *A. lancea* industry; common issues include excessive use of chemical fertilizers and pesticides, and continuous cropping obstacles. In addition, the growth and development of *A. lancea* is restricted by high temperatures above 30°C (Guo et al. 2005; Yan et al. 2010). *A. lancea* growth is hampered at elevated temperatures by a combination of root rot and a drop in net photosynthetic rate and stomatal conductance in leaves (Zhang et al. 2011; Li et al. 2015; Wang et al. 2016), leading to reduced production and economic losses. Because PGPBs have the capacity to promote plant growth and increase resistance to disease and heat (Mendes et al. 2013; Liu et al. 2017), research on PGPBs related to *A. lancea* could facilitate sustainable development of the *A. lancea* industry.

Medicinal plants crop contains secondary metabolites that can act as active ingredients in medicines; thus, the yield and content of secondary metabolites need to be specifically considered during the cultivation process. The content and composition of volatile oils are the standard parameters used to assess the quality of *A. lancea* (Guo et al. 2002). A previous study found that an endophytic *Pseudomonas fluorescens* not only promoted *A. lancea* growth, but also promoted the accumulation of volatile oils (Zhou et al. 2016). Another study reported that *Acinetobacter* sp. could promote more volatile oil components in *A. lancea*, but did not affect biomass (Wang et al. 2015); however, research on PGPBs of *A. lancea* is limited (Zhou et al. 2015; Zhou et al. 2016). Currently, the composition of the microbial community associated with the root of *A. lancea* remains unclear. Identifying PGPBs that can symbiotically promote the growth of *A. lancea*, promote resistance to pathogens and high temperature, and promote the accumulation of volatile oils, could improve the quality and yield of this crop. Therefore, the present study aimed to investigate the effects of soil microorganisms on the growth and content of secondary metabolites of *A. lancea*, as well as the composition of its endophytic and rhizosphere microbial community under different temperatures.

Material And Methods

Plant material

A. lancea plantlets were induced by buds of young stems obtained by the germination of wild *A. lancea* seeds collected from the Taurus Mountains, Jintan City, Jiangsu Province. Initially, full-grained wild seeds were carefully selected and washed thrice with soap water and then running tap water, respectively. The surface of the seeds was drained with absorbent paper. Thereafter, the remaining steps were carried out under aseptic conditions. The seeds were surface-sterilized by immersing in 75% ethanol for 30 s and then washed four times by soaking in sterile distilled water. They were immersed in 2% sodium hypochlorite for 15 min, then in 0.1% mercury chloride solution for 4 min, and finally rinsed five times in sterile distilled water. Eventually, the sterile surfaces of the seeds were dried with sterilized absorbent paper and placed on MS medium to accelerate germination (Wang et al. 2014).

The buds (approximately 2–3 cm long) of young stems of the germinated seedlings were intercepted and placed on 100 mL MS solid medium containing 100 mL distilled water, 0.02 mg naphthalene acetic acid (NAA), 0.2 mg 6-benzyladenine (6-BA), 3 g sucrose and 0.78% agar, to induce bud differentiation in 750 mL tissue culture flasks. The medium pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min. Four weeks after differentiation, the newborn axillary buds were separated and placed in the rooting medium (1/2 MS) comprising 1.0 mg/L NAA, 30 g/L sucrose, and 7% agar. All *in vitro* cultures were maintained in a growth chamber (23 ± 2 °C entire day, with a light intensity of 1,500–2,000 lx and a photoperiod of 12 h) and sub-cultured every 4 weeks (Wang et al. 2012; Wang et al. 2014).

Experimental design

Soil microorganisms: Native soil collected from the soil beneath forests of the mountain slopes (31° 36′ 18″ N, 119° 6′ 48″ E; the habitat of wild *A. lancea*) in Lishui District, Jiangsu Province, was used to prepare a soil suspension comprising the entire microbial community; 10 g of soil was dissolved into 100 mL sterile distilled water and mixed on a shaker at a rotating speed of 220 r/min for 10 min.

Sterile nutrient soil: The nutrient soil (thickness, 6 cm) was added into a 750-mL tissue culture flask and then placed in a high-pressure steam sterilization pot at 121 °C for 60 min.

Thereafter, *A. lancea* plantlets with short roots, grown on rooting medium for four weeks, were transplanted to the sterile nutrient soil. Nine days later, the new roots were observed from the bottom of the bottle. Subsequently, 5 mL soil suspension was inoculated around the roots of the plantlets (see Supplementary Fig. 1). In the present study, we set two temperature conditions of 23 ± 2 °C and 30 ± 2 °C. The 23 °C condition is a common temperature for plant culture in the laboratory. As high temperatures > 30 °C in the laboratory are likely to cause the death of *A. lancea* plantlets, the critical temperature of 30 °C was selected as the high temperature treatment. Fifteen repetitions of plantlets inoculated with soil suspension and 15 repetitions of plantlets inoculated with sterilized soil suspension were placed in a greenhouse (23 ± 2 °C entire day, with a light intensity of 1,500–2,000 lx and 12 h of daylight). N indicates normal temperature (23 °C) and free-microbe treatment group. N+B depicts inoculated microorganisms at normal temperature. Another 15 repetitions of plantlets inoculated with soil suspension and 15 repetitions of plantlets inoculated with sterilized soil suspension were placed in an

artificial climatic incubator (30 ± 2 °C for the entire day, with a light intensity of 1,500–2,000 lx and 12 h of daylight). H indicates high temperature (30 °C) and free-microbe treatment group. H+B indicates inoculated microorganisms at high temperatures. Three repetitions of sterile nutrient soils that were inoculated with sterilized soil suspension were placed in a greenhouse with a 23 °C temperature as the primary soil (CK). Simultaneously, nine repetitions of sterile nutrient soil without transplanting plantlets were placed in the greenhouse (23 ± 2 °C) after inoculation of the soil suspension. NBS indicates control soil inoculated with soil microorganisms at 23 ± 2 °C. Thereafter, another nine repetitions of sterile nutrient soil without transplanting plantlets were placed in the greenhouse (30 ± 2 °C) after inoculating the soil suspension. HBS presents control soil inoculated with soil microorganisms at 30 ± 2 °C. All the aforementioned treatment groups were sampled after 30 days and sample measurements were recorded.

Sample collection

All samples were collected and measured 30 days after inoculation. A total of 3 CK soil samples, each containing a repeat, were used for testing primary soil chemical properties. Nine NBS repetitions were randomly divided into three treatment groups with three repetitions each. Three duplicated soils were blended and used as a NBS for soil chemistry and microbiological testing. Nine HBS were sampled and tested in the same way. Fifteen repetitions were randomly divided into three treatment groups with five plantlets each. First, the plantlets were pulled from the soil and excess loose soil was shaken off. The soil tightly adhered to the roots surface was removed and collected as rhizosphere soil. Five rhizosphere soils from each treatment group were combined as one rhizosphere soil sample. NRS and HBS represent the rhizosphere soil in which plantlets were cultivated at normal temperatures and high temperatures, respectively, without microbial inoculation. NBRS and HBRS indicate the rhizosphere soil in which plantlets were cultivated at normal temperatures and high temperatures, respectively, with microbial inoculation. Thereafter, the roots of these plantlets were cleaned with sterile water, and then surface-dried with sterile absorbent paper for measuring the fresh weight. Second, each plantlet was weighed and a cut was made at the junction of the root and stem. The fresh weight of the whole plant minus the fresh weight of the root was used as the fresh weight of the stems and leaves. The roots were carefully straightened and the length was measured with a vernier caliper. The ratio of fresh weight of stems and leaves to the fresh weight of the roots was used as the root-shoot ratio. Third, two roots were removed from the roots of all plantlets and combined according to the aforementioned random grouping to detect the composition of endophytic bacterial diversity. Mixed root tissues from each grouping were placed in clean 50 mL conical tubes and pre-rinsed three times with sterile distilled water. The washed roots were then treated with 70% ethanol for 10 min, followed by a treatment with 2.5 % sodium hypochlorite and sonication for an additional 10 min. The samples were then drained and rinsed with sterile distilled water for three times. To check for surface sterility, 100 µL of the final rinsed solution was plated in Potato Dextrose Agar (PDA) and Nutrient agar (NA) and this resulted in zero colonies. Similarly, two other roots were removed and combined as above to detect the expression of key enzyme genes. Tissue samples and soil samples used for the detection of endophytic and rhizosphere bacteria were cooled in liquid nitrogen immediately after sampling, and then stored at -80℃ for later use. The remaining roots were

mixed accordingly to identify the contents of the four volatile oils including hinesol, β-eudesmol, atractylon, and atractylodin. Eventually, the root dry weight was measured after the roots used to detect the volatile oils had been dried.

Analysis of content of four volatile oil and key enzyme gene expression

The content of hinesol, β-eudesmol, atractylon, and atractylodin was measured via gas chromatography mass spectrometer (GC-MS). The rhizomes of different treatment groups used to detect volatile oil were dried in a freeze-dryer for approximately 72 h until they attained a constant weight, and were then ground into powders. The volatile oil was extracted with a solid–liquid ratio of 1:4, that is, 0.1 g powder was soaked in 0.4 mL n-hexane. After 15 min of sonication (60 Hz), the mixture was centrifuged for 5 min at 5000 × *g* at 4 °C. The supernatant was separated, filtered through a 0.22 μm microporous filter, and stored in a brown sample vial at 4 °C for GC-MS analysis (Yang et al. 2019; Yuan et al. 2019). The aforementioned components were quantified using a Trace 1310 series GC with a TSQ8000 MS detector (Thermo Fisher Scientific Co. Ltd, Waltham, Massachusetts, USA). A TR-5ms capillary column (30 m 3 0.25 mm i.d., DF = 0.25 mm, Thermo Fisher Scientific) was used for GC-MS analysis. A few adjustments were made to the program, according to the method of Li et al. (2018), as follows: the injected sample (1 μL) was separated with a Helium flow rate of 1 ml/min with a temperature program of 2 min at 120 °C, followed by a gradient from 120 °C to 240 °C at 5 °C/min, and held at 240 °C for 5 min. The injector and detector temperatures were set at 240 °C and 350 °C, respectively. MS operating conditions were as follows: the MS ionization mode indicated the electron impact ion source (EI) at 230 °C, with an acceleration voltage of 70 eV. The interface temperature was 240 °C and the total ion current was recorded for a mass range of 40–500 amu (Li et al. 2018; Yang et al. 2019; Yuan et al. 2019). The contents of four volatile oils in each sample were quantitatively determined by the standard curves (see Supplementary Table 1).

In plants, the precursors of terpenoids can be produced through mevalonate (MVA) and methylerythritol-4-phosphate (MEP) pathways (Vranová et al. 2013). HMGR (3-hydroxy-3-methylglutaryl-coenzyme A reductase) and DXS (1-deoxy-D-xylulose 5-phosphate synthase) are the first rate-limiting enzymes in the MVA and MEP pathways, respectively (Zhao et al. 2010). Moreover, sesquiterpene biosynthesis requires a key enzyme, farnesyl diphosphate synthase (FPPS) (Cane 1999; Shakeel et al. 2016). Real time quantitative reverse transcription PCR (real time qRT-PCR) was performed to detect the expression levels of key enzyme genes of plantlets in different groups, including HMGR, FPPS, and DXS (Liu et al. 2007; Deng et al. 2017; Jiang et al. 2017; Lu et al. 2019). Ribosomal protein 18 (18S) was used as an internal reference (Jiang et al. 2017). Primers for the three selected genes are listed in supplementary Table 2; they were synthesized by Beijing Ruibo Biotechnology Co., LTD, Beijing, China. The total RNA extracted from the roots was used to detect the expression of key enzyme genes using a quick RNA isolation kit (Hua Yue Yang biotechnology, Beijing, China). The results of agarose gel electrophoresis revealed high quality total RNA (see Supplementary Fig. 2); therefore, further tests could be carried out. Approximately 2

μg of total RNA was reverse-transcribed into cDNA using a kit (Prime Script One Step RT Reagent Kit; Takara, Dalian, China) (Jin et al. 2019). The reaction system of real time-qPCR was as follows: 10 μL SYBR Premix Ex Taq (2×), 1 μL PCR forward primer (10 pmol/μL), 1 μL PCR reverse primer (10 pmol/μL), 2 μL cDNA template, replenished with ddH_2O to 20 μL. Real time-qPCR was performed at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 40 s (Lu et al. 2019).

Analysis of soil chemical properties

Five physical and chemical indices of soil were detected, namely, pH, nitrate nitrogen, ammonium nitrogen, available phosphorus, and available potassium, respectively. The pH values of soil were measured using the glass electrode method (soil and water suspension v/v 1:2.5) (Iso 1994). Soil nitrate nitrogen, soil ammonium nitrogen, available phosphorus, and available potassium contents were determined via ultraviolet spectrophotometry (GB/T 32737-2016), indophenol blue colorimetry (LY/T 1228-2015), colorimetry (NY/T 1121.7-2014), and the extraction-molybdenum-antimony anticolorimetric method (NY/T 889-2004), respectively.

DNA extraction, 16S amplicon sequencing, and bioinformatics analysis

DNA extraction and 16S amplicon sequencing

The total bacterial DNA extracted from 100 mg root tissues, 150 mg rhizosphere soil, and 150 mg control soil using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), was used for amplification and sequencing of the 16S rRNA, targeting the variable V3–V4 regions (Xu et al. 2016; Perez-Jaramillo et al. 2019), thereby resulting in amplicons of approximately 460 bp. The gene primers were 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Error-correcting barcodes were added to both forward and reverse primers (Hamady et al. 2008). Extracted DNA was detected by 1% agarose gel electrophoresis. PCR reaction was carried out on a GeneAmp 9700 PCR system (Applied Biosystems, Foster City, CA, USA). The total volume of each PCR amplified reaction system was 25 μL, including 1 μL DNA template, 0.5 μL forward primer, 0.5 μL reverse primer, 0.25 μL bovine serum albumin, 12.5 μL 2× DreamTaq Green PCR Master Mix (Thermo Scientific, USA), replenished with ddH_2O to 25 μL. Setting three replicates for each reaction, PCR was carried out as follows: 95 °C for 3 min, followed by 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min. Three technical repeats of one sample were mixed into a single PCR product. The products were separated via 2% agarose gel electrophoresis and purified using a Qiagen PCR purification kit (Qiagen, Hilden, Germany). Furthermore, the purified products were quantified with Pico Green using a QuantiFluorTM-ST Fluorometer (Promega Biotech, Beijing, China) and were then pooled at equal concentrations. Thereafter, the amplicons were sequenced in an Illumina MiSeq platform (San Diego, CA, USA) at Shanghai Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China.

Bioinformatics analysis

First, paired-end (PE) reads obtained by MiSeq sequencing were spliced according to their overlap relations using FLASH (Magoč and Salzberg 2011), and quality-filtered using Trimmomatic (Bolger et al. 2014). All sequences were divided into operational taxonomic units (OTUs) with 97% similarity or greater using UPARSE software (version 7.0) (Edgar 2013), and a majority consensus taxonomy was obtained for each OTU. Singletons were removed from the datasets to minimize the impact of sequencing artifacts (Dickie 2010). Chimeric sequences were identified and removed using UCHIME (Edgar et al. 2011). In order to obtain the species classification information corresponding to each OTU, the RDP classifier algorithm (https://sourceforge.net/projects/rdp-classifier/) was applied to compare the OTU representative sequences with the Silva database (SSU138) for taxonomic analysis using confidence threshold of 70%. Among these, chloroplasts and mitochondrial sequences were removed. The bacterial community diversity and richness were demonstrated using the Shannon index, Simpson index，Chao index, and ACE index using Mothur v.1.30.1 (http://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity). Student's *t*-test was used to compare the significance of the index differences between groups. The significance level to threshold (P value) was set at 0.05. The relative abundance bar of bacteria at the phylum and genus levels was visualized using R language tools (v.3.3.1). Analysis of Principal Coordinates Analysis (PCoA) between root tissue samples was performed using QIIME (version 1.9.1) based on unweighted-Unifrac distance matrix. A non-metric multidimensional scaling (NMDS) ordination to illustrate the clustering of control soil and rhizosphere soil bacterial community composition variation was conducted using the Vegan software based on the Bray-Curtis distance of genus. The Student's *t*-test was used to examine differences in bacterial composition between the two groups. The relation between relative abundance of genus and the contents of four volatile oils or soil chemical properties was performed using Spearman's correlation analyses.

Statistical analysis

In the present study, biomass, volatile oil, key enzyme gene expression, and soil physicochemical data as aforementioned were recorded and processed by Excel (Office 2019). Thereafter, GraphPad Prism 8.0.1 (GraphPad Software Inc., USA) was used for rendering graphics. One-way ANOVA of plant biomass, the contents of four volatile oils, key enzyme gene expression, and soil chemical properties were determined using IBM SPSS Statistics 19.0 (SPSS, Chicago, IL, USA). Significance was calculated by Tukey's test ($p < 0.05$). Results are expressed as mean ± standard deviation (S.D.). $P<0.05$ and $P<0.01$ were considered to be statistically significant and extremely significant, respectively.

Results

Effects of soil microorganisms on biological indices of *A. lancea*

As shown in Figure 1 (a, d, e), high-temperature significantly inhibited the total fresh weight, fresh root weight and root-shoot ratio of plantlets without soil microbial inoculation ($P < 0.05$). However, the inhibition of high-temperature on the above indices were alleviated or relieved after soil microbial inoculation. Moreover, the addition of soil microorganisms always significantly promoted root length, fresh root weight, root-shoot ratio and dry root weight of plantlets in the inoculated group compared with the non-inoculated group at either high-temperature or normal temperature (N+B *vs* N and H+B *vs* H in Fig. 1b, d, e, f) ($P < 0.05$). Surprisingly, inoculation of microorganisms decreased the biomass of the above-ground of the plantlets, while increased the biomass of the under-ground part. High-temperature, however, reduced the fresh weight of both the upper and underground parts of the plantlets simultaneously (Fig. 1c, d). In short, high temperature had a strong inhibitory effect on the growth and development of plantlets, and microorganisms could not only promote the growth of plantlets, but also offset the adverse effects of high temperature.

Effects of soil microorganisms on the contents of four volatile oils in *A. lancea*

As shown in Figure 2a, three sesquiterpenoids, hinesol, β-eudesmol, and atractylon, were absent or present in low quantities in the control groups N and H. However, all three were detected and increased significantly at different temperatures after microbial inoculation ($P < 0.05$). The hinesol and β-eudesmol levels ranged from 15 to 23 μg/g, and atractylon level ranged from 100 to 150 μg/g. Moreover, the contents of hinesol and atractylon in H+B group were slightly higher than those in N+B group. Furthermore, compared with the control groups N and H, the atractylodin content in N+B and H+B increased by 4.4 times and 3.6 times, respectively. The aforementioned data indicated that the microorganisms could significantly increase the accumulation of the volatile oils in *A. lancea* ($P < 0.05$), while high-temperature could indirectly regulate the content of sesquiterpenoids by microorganisms.

Expression analysis of key enzyme gene in sesquiterpenoid synthesis pathway in *A. lancea*

Figure 2b illustrates that the expression level of the critical enzyme DXS gene was deficient, which is consistent with the reported results. The expression level of the DXS gene was highest in leaves and lowest in rhizomes (Deng et al. 2017). This indicated that the synthetic pathway of sesquiterpenes in *A. lancea* may not contain the MEP pathway. In contrast, the expression levels of the HMGR gene and FPPS gene were higher in the N+B and H+B groups than those in the control group (N and H). We also found that the expression levels of HMGR and FPPS in H+B group were higher than those in N+B group, which is consistent with the content of sesquiterpenoids. These results suggested that microorganisms were the key factor to increase MVA pathway gene expression level.

Analysis of soil chemical properties

First, we compared the effects of microorganisms and high temperature on the chemical properties of soil (CK, NBS and HBS) in the absence of plants. Whether at normal temperature or high temperature, microorganisms markedly reduced the pH and increased the content of NH_4^+-N and available P of soil (NBS *vs* CK or HBS *vs* CK; $P < 0.001$ or $P < 0.05$). It is also beneficial to the increase of NO_3^--N. (Fig. 3a–d). Next, we compared the effects of microbes and high temperature on chemical properties of rhizosphere soil. In the absence of microorganisms, the plantlets could lower the pH and increased NO_3^--N and NH_4^+-N levels in the rhizosphere soil (NRS *vs* CK and HRS *vs* CK) (Fig. 3a, b). Although there was no difference between the five indexes in rhizosphere soil inoculated with microorganisms and those in uninoculated rhizosphere soil, the microorganisms had the same synergistic function on soil chemistry as plants, which was beneficial to the absorption and utilization of soil nutrients by *A. lancea*. Moreover, high temperatures facilitated both processes separately. In contrast, the microbes, high temperature, or plantlets did not affect the K content in the soil (Fig. 3e).

Taxonomic features of the endophytic bacteria of *A. lancea* and their correlation with volatile oils contents

Taxonomic features of endophytic bacteria of *A. lancea*

On day 30 post inoculation, we performed 16S rRNA amplicon sequencing of root tissues, rhizosphere soil, and control soil samples. Approximately 1.19 million high-quality sequence tags were generated for the 16S sequencing samples. Eventually, 342 OTUs were obtained from the root tissue samples, and taxonomic characteristics of each OTU were achieved through taxonomic annotations. These OTUs belong to 19 phyla and 226 genera. Figure 4 (a–d) illustrates that the plantlets were not utterly sterile. Figure 4a and 4b shows that high temperature reduced the diversity and richness of root endophytes at the OTU level without microbial inoculation. After microbial inoculation, the diversity of H+B was similar to that of N+B, with a slight decrease. In contrast, the species richness of H+B group was significantly lower than that of N+B group ($P < 0.05$).

The endophytic bacteria of the plantlets were mainly comprised of the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes, and the total relative abundance reached more than 95% on all accounts (Fig. 4c). Furthermore, without microbial inoculation, the composition of the endophytic bacterial community was affected by high temperatures. Compared with N, Firmicutes, Actinobacteria, Bacteroidetes, and Acidobacteria in H were increased, whereas the relative abundance of Proteobacteria and Chloroflexi was decreased. After inoculation, the relative abundance of Proteobacteria in N+B and H+B groups increased compared with that in N and H groups, whereas the relative abundance of Firmicutes, Actinobacteria, Bacteroidetes, and Acidobacteria decreased. At the genus level (Fig. 4d), the H group mainly comprised *Enterococcus* and *Cutibacterium*; whereas the N group mainly comprised *Enterococcus*, *Burkholderia-Caballeronia-Paraburkholderia (Paraburkholderia)*, *Cutibacterium*, *Ralstonia*, *Acinetobacter*, *Provotella_1*, and *Streptococcus*. Nevertheless, the bacterial community in group H+B weakened under the influence of high temperature

and achieved a composition similar to that in group N+B after inoculation. Only two species of bacteria showed significant difference between N+B and H+B groups (supplementary Fig. 3a). PCoA of unweighted UniFrac distances between samples revealed that exogenous microorganisms were the primary factor affecting the structure of endophytic bacteria (PC1=19.04%), followed by temperature (PC2=14.07%) (Fig. 5a). Student's *t*-test revealed that the relative abundance of *Rhodococcus* increased significantly in group N+B compared with that in group N (Fig. 6a). In the H+B group, compared with the H group, the relative abundance of the *Enterococcus* decreased significantly, whereas that of the *Paraburkholderia, Ralstonia, Paenibacillus,* and *Dongia* increased significantly, with *P* values of 0.01, 0.001, 0.01, and 0.001, respectively (Fig. 6b). According to the Student's *t*-test results, the relative abundance of *Paraburkholderia*, *Ralstonia*, *Bradyrhizobium*, *Paenibacillus*, *Bacillus*, and *Rhodococcus* was significantly increased when the groups N and H were treated as non-inoculation groups and N+B and H+B as inoculation groups (see supplementary Figure 3b). The aforementioned data indicate that exogenous microorganisms could reduce the interference of high temperature on endophytic bacteria and also provide sources for endophytic bacteria.

Spearman correlation analysis between endophytic bacteria from roots of *A. lancea* and four volatile oils

Figure 7 illustrates the Spearman correlation analysis between the 30 most abundant endophytic bacteria from the roots of *A. lancea* and the four volatile oils. *Paraburkholderia*, *Paenibacillus* and *Bradyrhizobium* exhibited significant or extremely significant positive correlations with the four volatile oils in *A. lancea*. In addition, *Rhodococcus* and *Ralstonia* tended to positively correlated with the four volatile oil components. In contrast, *Escherichia-Shigella*, *Cutibacterium*, *Enterococcus*, norank_f__Muribaculaceae, and *Stenotrophomonas* revealed different degrees of negative correlation with the four volatile oils. In conclusion, endophytic bacteria enriched by *A. lancea* could positively promote the accumulation of volatile oils.

Taxonomic features of the rhizosphere bacteria of *A. lancea* and their correlation with soil chemical properties

Taxonomic features of the rhizosphere bacterial of *A. lancea*

In total, 1,522 OTUs were obtained from the control soils (NBS, HBS) and rhizosphere soils (NBRS, HBRS). Taxonomic annotation revealed that these OTUs belonged to 26 bacteriophyta and 496 bacteriological genera. As shown in Figure 8a, under different temperatures, Simpson index showed that the bacterial diversity of rhizosphere soil inoculated with microorganisms was lower than that of control soil, and NBRS was significantly lower than NBS ($P < 0.05$). However, according to the Chao index, no significant difference was observed in species richness between the rhizosphere and control soils (Fig. 8b). In Figure 8c, the rhizosphere bacteria of plantlets mainly comprised Proteobacteria, Patescibacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia, and Acidobacteria. Compared with the control soil, the relative abundance of Proteobacteria and Bacteroidetes in rhizosphere soil decreased, whereas that of

Patescibacteria increased. At the genus level, high-temperature significantly reduced the relative abundance of *Sphingomonas* and *Chitinophaga* abundance in the control soil ($P < 0.05$ and $P < 0.01$, respectively) (supplementary Fig. 4a). Nine bacterial genera among the top 15 in group NBRS were significant differences from those in group NBS (Figs. 8d and 9a). Among nine, norank_o_Saccharimonadales and *Novosphingobium* were significantly enriched in the rhizosphere of plantlets in group NBRS and HBRS (Fig. 9a and b). No significant difference was observed in rhizosphere bacteria under different temperatures (supplementary Fig. 4b). Based on bray-Curtis distance, NMDS analysis of bacterial community composition showed that there were differences in community composition between rhizosphere bacteria and control soil bacteria, which indicated that *A. lancea* plantlets could form a unique rhizosphere community. High temperature affected the composition of control soil bacterial community, but the composition of rhizosphere bacterial community was more similar and less affected by high temperature (Fig. 5b).

Spearman correlation analysis between rhizosphere bacteria of *A. lancea* and soil chemical properties

Spearman correlation analysis was performed on the rhizosphere bacteria and soil nutrients at the genus level (Fig. 10). The results indicated that most of the bacteria in the top 30 relative abundance genera were positively correlated with NO_3^--N, NH_4^+-N, available P, and available K in the soil. Only *Massilia* exhibited a significant positive correlation with available P. Conversely, numerous bacterial genera such as *Candidatus_Solibacter* and *Gemmatimonas* that are positively correlated with NO_3^--N, NH_4^+-N, available P, and available K were negatively correlated with soil pH.

Discussion

In the present study, we found that soil microorganisms could effectively promote plant growth and development at both normal temperature and high temperature, including root elongation growth and accumulation of organic matter; moreover, they could increase the accumulation of volatile oils in the root of *A. lancea*. Roots are an important area where plant and soil interactions occur (Tringe et al. 2005; Zhang et al. 2009). The diversity analysis of the endophytic and rhizosphere bacteria revealed that the relative abundance of *Burkholderia-Caballeronia-Paraburkholderia*, *Ralstonia*, *Bradyrhizobium*, *Paenibacillus*, *Bacillus*, and *Rhodococcus* increased in the root of *A. lancea* plantlets at high or normal temperature (Fig. 4d), and these bacteria were significantly enriched in the roots of *A. lancea* without considering the effect of temperature (supplementary Fig. 3b). This result is consistent with field sampling (unpublished), indicating that *A. lancea* can specifically recruit these bacteria to form specific endophytes. Endophytic bacteria exhibit numerous functions in plants such as plant production characteristics as well as their stress response mechanisms (Dini-Andreote 2020). These bacteria can produce indoleacetic acid (IAA), cytokinins, gibberellins, and other plant hormones or regulate the production of internal hormones to promote plant growth (Spaepen and Vanderleyden 2011; Hardoim et al. 2015; Santoyo et al. 2016). Furthermore, they can produce siderophores to increase the absorption of iron by plants in iron-limited conditions (Ahmed and Holmstrom 2014; Hardoim et al. 2015). In addition,

phosphorus-solubilizing bacteria can dissolve the mineral phosphorus in the soil to produce phosphorus, which can be used by plants (Armando et al. 2009; Oteino et al. 2015). Both *Burkholderia-Caballeronia-Paraburkholderia* and *Ralstonia* belong to Burkholderiaceae; however, the function of *Burkholderia-Caballeronia-Paraburkholderia* in plants remains unclear. Nevertheless, numerous studies have reported the phosphorus-solubilizing, nitrogen-fixing, degradation and biotransformation of organic compounds, and growth stimulating effects of *Burkholderia, Caballeronia*, and *Paraburkholderia* in plants (Morya et al. 2020; Paulitsch et al. 2020; Sadauskas et al. 2020; Tapia-Garcia et al. 2020b; Yang et al. 2020; Ravi et al. 2021). Therefore, *Burkholderia-Caballeronia-Paraburkholderia* should also promote growth and increase the accumulation of volatile oils in *A. lancea*. The 16S rRNA sequences of *Ralstonia* were identified to have 99.0% similarity with *Ralstonia solanacearum* in the V3–V4 region. *Ralstonia solanacearum* is one of the most important bacterial plant pathogens and causes bacterial wilt on various crops (Palleroni and Doudoroff 1971; Chen et al. 2001; Elphinstone 2005). Six phenotypes have been defined for the interaction of *Ralstonia solanacearum* with the host. The intermediate phenotype corresponds to the potential infection of the plant, that is, bacterial colonization in the xylem with little or no wilting symptoms (Lebeau et al. 2011). The *Ralstonia* genus also possesses beneficial bacteria such as *Ralstonia taiwanensis* sp. nov. a β-proteobacteria that forms root nodules in the Leguminosae roots and fixes nitrogen (Chen et al. 2001; Chen et al. 2003). In the present study, *Ralstonia* was significantly enriched in the roots of *A. lancea*, but wilting of plantlets was not observed. *Ralstonia* does not exhibit pathogenic effects on *A. lancea* (Wang et al. 2016); moreover, its pathogenic effect and role in the growth and development of *A. lancea* remains to be verified.

Notably, *Bradyrhizobium* is a genus of plant growth-promoting rhizobacteria (PGPRs) which promotes nitrogen acquisition in legumes and exists in the rhizosphere or roots of other plants and promotes their growth (Gonzalez et al. 2019; Greetatorn et al. 2019; Nguyen et al. 2020; Padukkage et al. 2020; Wasai-Hara et al. 2020; Zeffa et al. 2020). Numerous *Paenibacillus* species can promote crop growth directly via biological nitrogen fixation, phosphate solubilization, production of the phytohormone IAA, and release of siderophores that enable iron acquisition. Simultaneously, antimicrobials and insecticides can be produced to protect plants as these can trigger systemic resistance in plants and enhance disease resistance (Grady et al. 2016). Furthermore, *Bacillus* promotes plant growth, induces systematic resistance, and resists pathogenic bacteria (Saxena et al. 2020; Susic et al. 2020; Wang et al. 2020a; Wang et al. 2020b). The 16S rRNA sequences of *Rhodococcus* exhibited 100% similarity with *Rhodococcus erythropolis* in the V3–V4 region. *Rhodococcus erythropolis* can degrade N-acyl homoserine lactone (AHL) signals via a catabolic pathway to control the virulence of soft-rot pathogens (Barbey et al. 2018; Chane et al. 2019a; Chane et al. 2019b). In addition, it may produce IAA and siderophores, solubilize phosphorus, and promote plant growth and biomass accumulation (Mikolasch et al. 2016; Pacwa-Plociniczak et al. 2016). Spearman correlation analysis revealed that these beneficial bacteria were positively correlated with the four volatile oils to different degrees. This combination of endophytic bacteria in *A. lancea* is specific and has not been observed in other plants.

We found that the endophytic bacteria in the roots of *A. lancea* plantlets were mainly derived from the soil. The high abundance and diversity of soil microorganisms were conducive to the stability of

endophytes and resistance to external factors. A similar phenomenon has been observed in other plants (Wagner et al. 2016). Plants secrete up to 40% of photosynthetic products into the rhizosphere, resulting in a population density of microbes much higher than that in the surrounding soil, a phenomenon known as the rhizosphere effect (Bais et al. 2006). Recent studies reported that plants depend on rhizosphere microorganisms for nutrient acquisition, immune regulation, resistance to soilborne pathogens, and to enhance stress tolerance (Berendsen et al. 2012; Mendes et al. 2013). In the present study, *A. lancea* formed a unique rhizosphere. At normal temperature, norank_o_Saccharimonadales, *Mucilaginibacter*, norank_f_Micropepsaceae, and *Novosphingobium* were significantly enriched in the rhizosphere of *A. lancea*. Moreover, *Novosphingobium* contains various beneficial bacteria, which fix nitrogen, produce IAA, and resist salt stress (Rangjaroen et al. 2017; Vives-Peris et al. 2018). Unexpectedly, the relative abundance of *Sphingomonas*, *Burkholderia-Caballeronia-Paraburkholderia*, *Chitinophaga*, *Pararhizobium_Rhizobium*, and *Bradyrhizobium* in the rhizosphere of *A. lancea* was significantly reduced (Fig. 9a). But these bacteria have been reported to have the functions of nitrogen fixation, phosphate solubilization, and production of IAA (Bethlenfalvay et al. 1982; Chen et al. 2014; Asaf et al. 2018; Rilling et al. 2018; Luo et al. 2019; Luo et al. 2020; Morya et al. 2020; Sadauskas et al. 2020; Tapia-Garcia et al. 2020a); moreover, *Chitinophaga* produces various chitinases and effectively inhibits plant pathogenic fungi and root knot nematodes (Yin et al. 2013; Sharma et al. 2020). Although these beneficial bacteria appeared to be reduced in the rhizosphere of *A. lancea*, the microbial abundance was increased compared with that of the non-inoculated *A. lancea* and acted as a source of endophytes. Spearman correlation analysis results demonstrated that most rhizosphere bacteria were positively correlated with soil nutrients, which was beneficial to the absorption and utilization of soil nutrients by *A. lancea.*

The effect of temperature on *A. lancea* was verified in the present study. The biological phenotype of *A. lancea* revealed that when compared with group N, high temperature conditions could significantly inhibit the growth of *A. lancea*($P < 0.05$) (Fig. 1), and reduce soil pH by affecting soil microorganisms and *A. lancea*, and increase the content of NO_3^--N, NH_4^+-N, and available P. In addition, high temperature reduced the diversity and richness of endophytic bacteria in uninoculated soil. However, after the root inoculation of microorganisms in *A. lancea*, the influence of temperature on the diversity and richness of endophytic bacteria in *A. lancea* was weakened. The composition of N + B and H + B groups was similar (supplementary Figs. 4b and 5b), thereby indicating that the increase in microbial diversity and richness in soil could reduce the disturbance of high temperature and benefit the stability of endophytic bacteria in *A. lancea*. Therefore, for the stable development of the *A. lancea* cultivation industry, it is recommended to use environmentally friendly microbial preparations and increase PGPBs related to *A. lancea*. In addition, soil microorganisms include bacteria and fungi. In this experiment, we mainly detected the diversity of bacteria in the root and rhizosphere of *A. lancea*; however, whether *A. lancea* can recruit specific fungal groups remains to be clarified.

Conclusion

In the present study, soil microorganisms reduced the damage caused by high temperature to *A. lancea* seedlings; moreover, they promoted the growth and development of *A. lancea* and increases the accumulation of volatile oils. Simultaneously, soil microbes provided a source for endophytic bacteria, which was conducive for the stability of endophytic bacterial composition and reduced the interference associated with high temperature. In addition, *A. lancea* could establish specific bacterial communities in root and rhizosphere. Almost all bacteria enriched in the roots and rhizosphere of *A. lancea* were beneficial, with the exception of a potentially pathogenic bacterium called *Ralstonia.* The results of the present study could facilitate the development of PGPBs related to *A. lancea* and the safe and effective application of *A. lancea* in the cultivation industry.

Declarations

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Author contributions All authors contributed to the study conception and design. Material preparation and data collection and analysis were performed by Hongyang Wang, Daiquan Jiang, and Zengxu Xiang. The first draft of the manuscript was written by Hongyang Wang, and all authors commented on previous versions of the manuscript and authors read and approved the final manuscript. The authors declare that they have no competing interests.

Compliance with ethical standards

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Conflicts of interest There are no moral and ethical issues or conflicts to declare in this paper.

Availability of data and material Publicly available datasets were analyzed in this study. These data can be found in the NCBI database under accession numbers SRR13132034–SRR13132056.

Code availability The codes in this article are available on the free online platform of Majorbio Cloud Platform (www.majorbio.com).

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors approve publication of the final manuscript.

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Figures

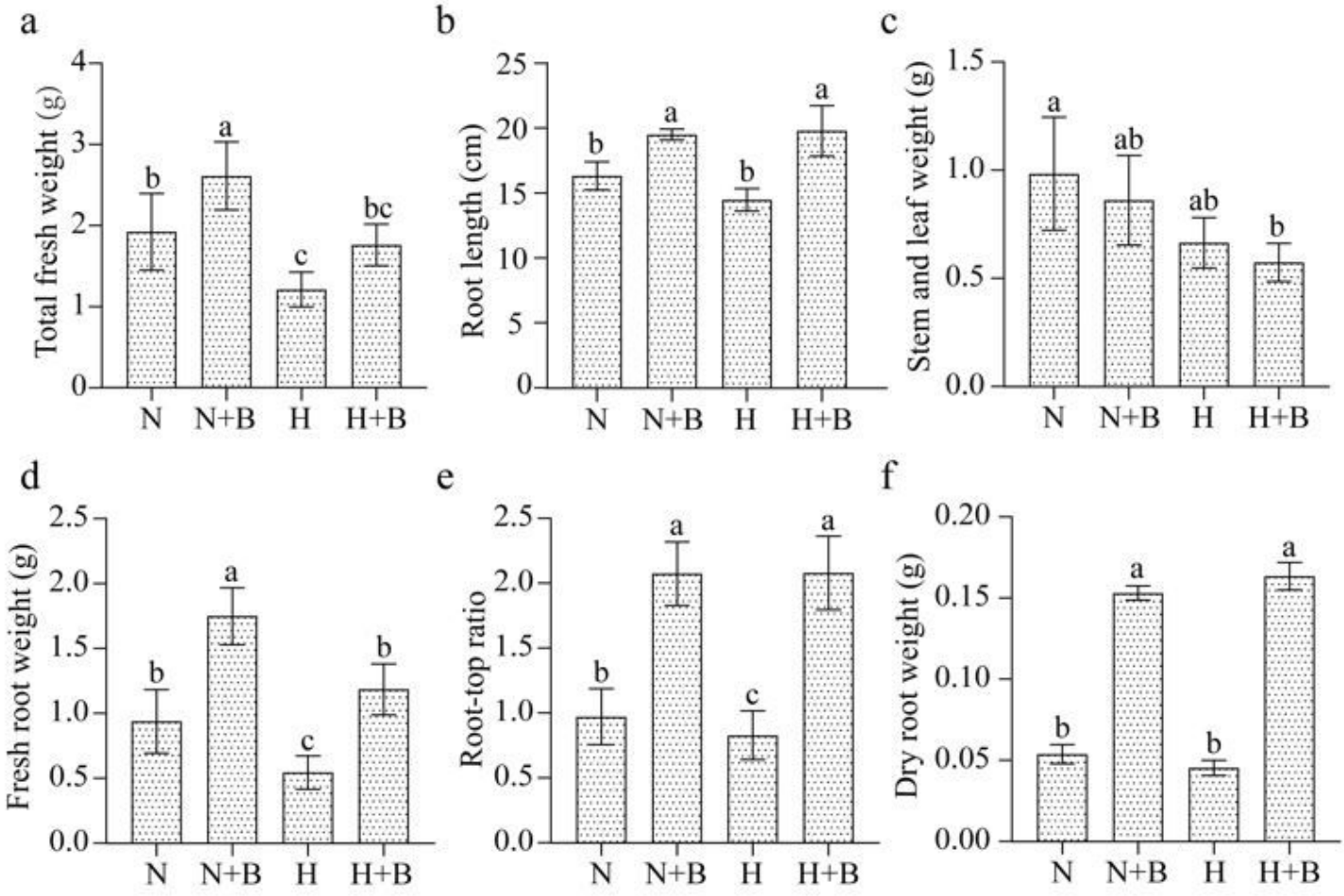

Figure 1

The effects of soil microorganisms and temperature on the biological indices of Atractylodes lancea plantlets. a Total fresh weight includes stems, leaves, and roots. b Root length of plantlets. b Fresh stem and leaf weight. d Fresh root weight. e Root-shoot ratio. f Root dry weight. Letters at the top of columns represent significant differences (one-way ANOVA, $P < 0.05$).

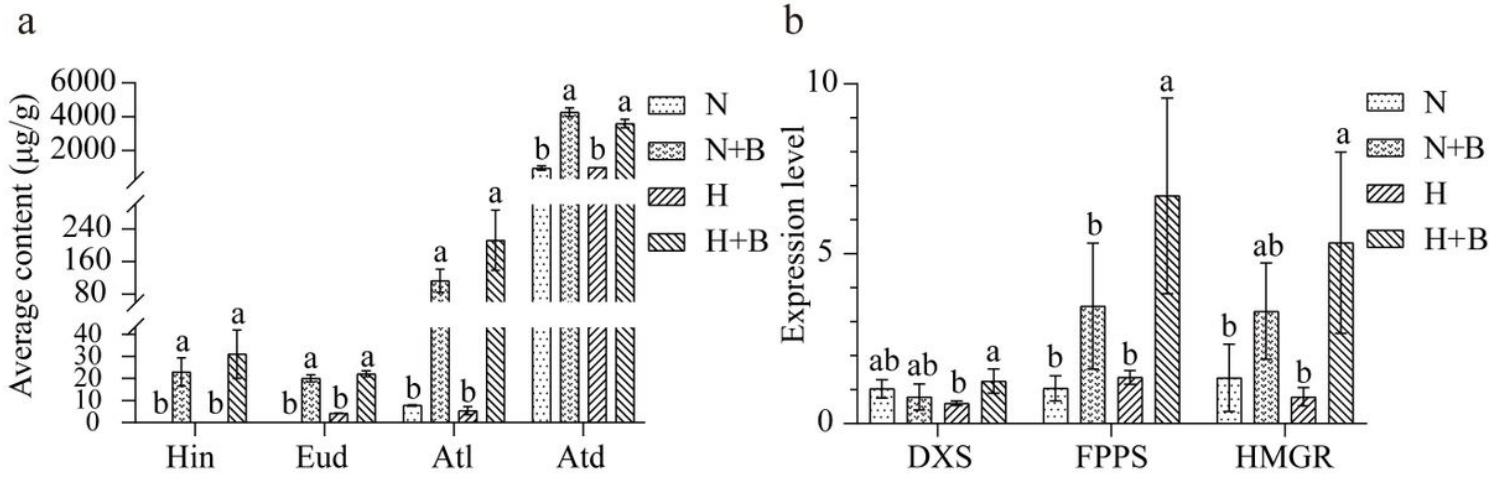

Figure 2

The contents of four volatile oils and expression levels of the key enzyme genes involved in synthesizing the volatile oil components of sesquiterpenes in the root tissues of Atractylodes lancea in different treatment groups. a The contents of four volatile oils in the root tissues in different treatment groups. b Key enzyme gene expression levels in the pathway related to sesquiterpenes synthesis. hinesol, hinesol; β-eudesmol, β-eudesmol; atractylon, atractylon; atractylodin, atractylodin. a, b, and c at the top of the column represent significant differences (one-way ANOVA, P < 0.05).

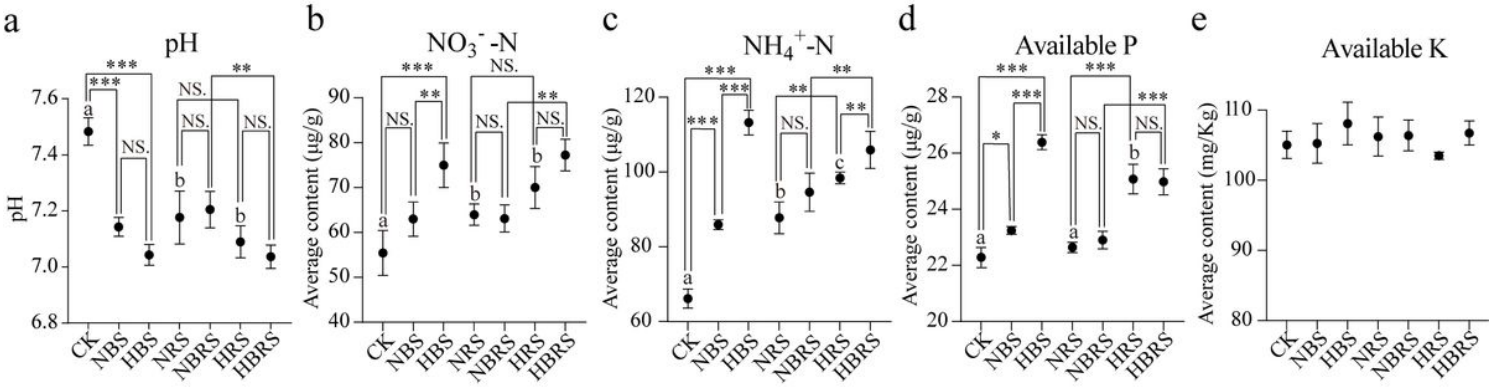

Figure 3

The effects of microbes and high temperature on five physical and chemical indices of soil. Effects of microbes and temperature on a soil pH, b soil NO3—N, c soil NH4+-N, d soil available P, and e soil available K. NS. indicates no significant difference (one-way ANOVA, P < 0.05). * indicates a significant difference (one-way ANOVA, P < 0.05). ** indicates an extremely significant difference (one-way ANOVA, P < 0.01). *** indicates an extremely significant difference (one-way ANOVA, P < 0.001).

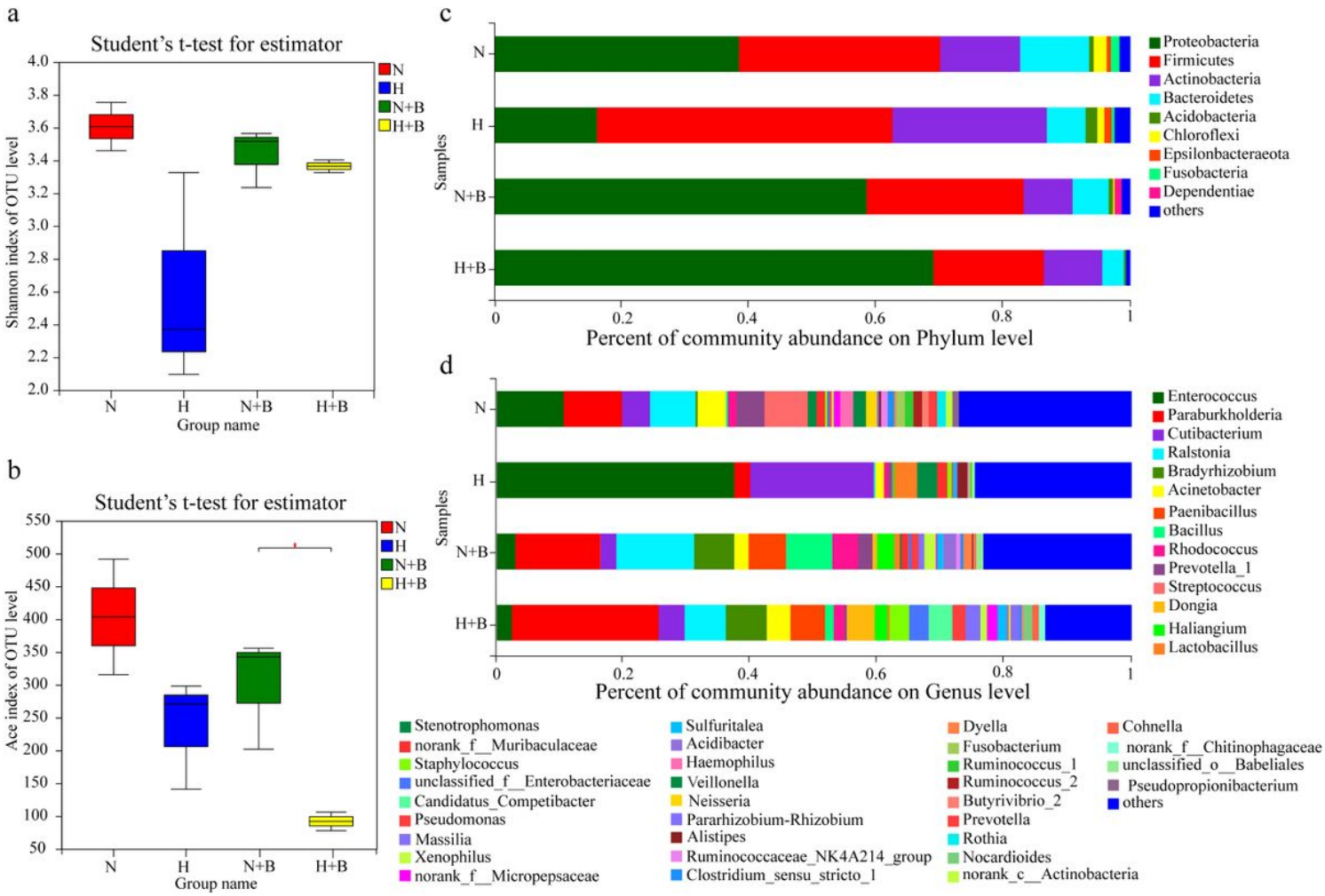

Figure 4

Comparative analysis of the alpha diversity and taxonomic distribution of 16S rRNA endophyte sequences from Atractylodes lancea plantlet root tissues in the inoculated and non-inoculated groups at normal or high temperature. a Shannon species diversity index at the OTU level. b ACE species richness index at the OTU level. c Phylum-level distributions in the root tissue samples based on 16S amplicon sequencing at normal temperature and high temperature. d Genus-level distributions in the root tissue samples based on 16S amplicon sequencing at normal temperature and high temperature. * indicates a significant difference (Student's t-test, $P < 0.05$).

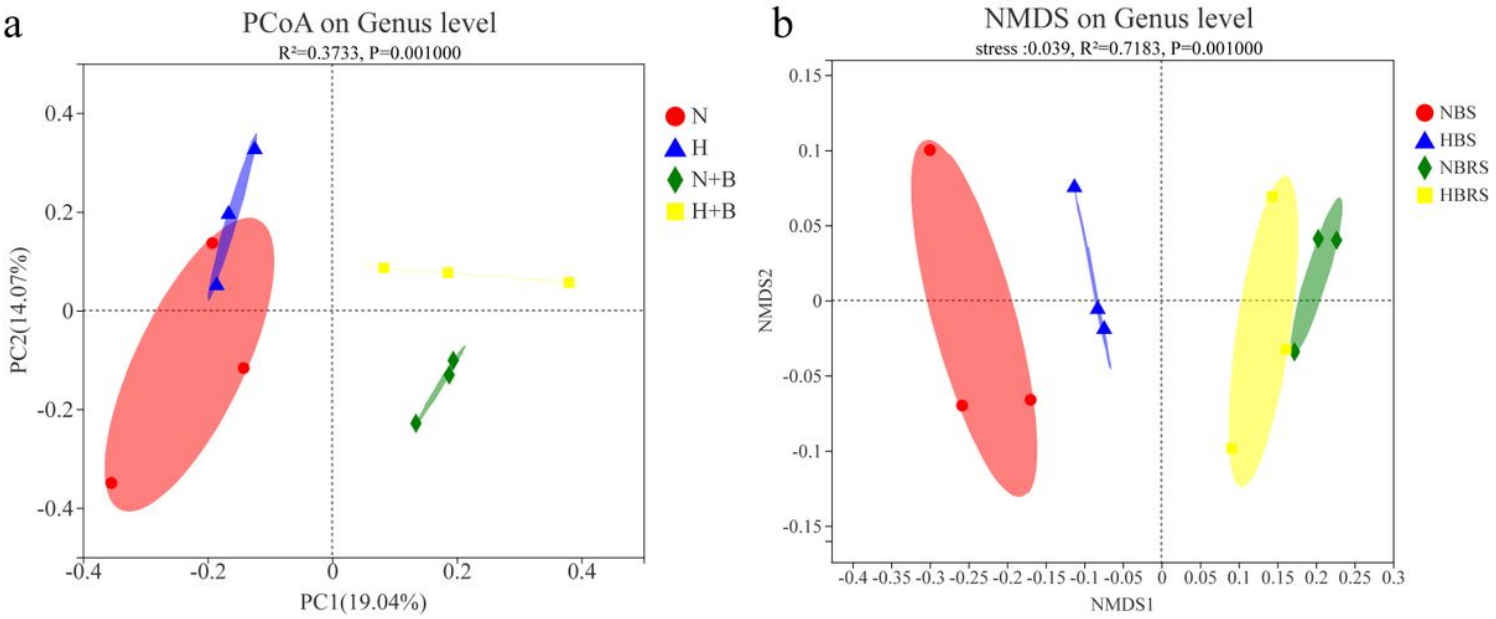

Figure 5

Beta diversity analysis of endophytic and rhizosphere bacterial communities. a PCoA of endophytic bacterial based on unweighted-Unifrac distance matrix of genus. b NMDS of control soil and rhizosphere soil bacterial based on the Bray-Curtis distance of genus. PCoA and NMDS analysis were based on three biological replicates.

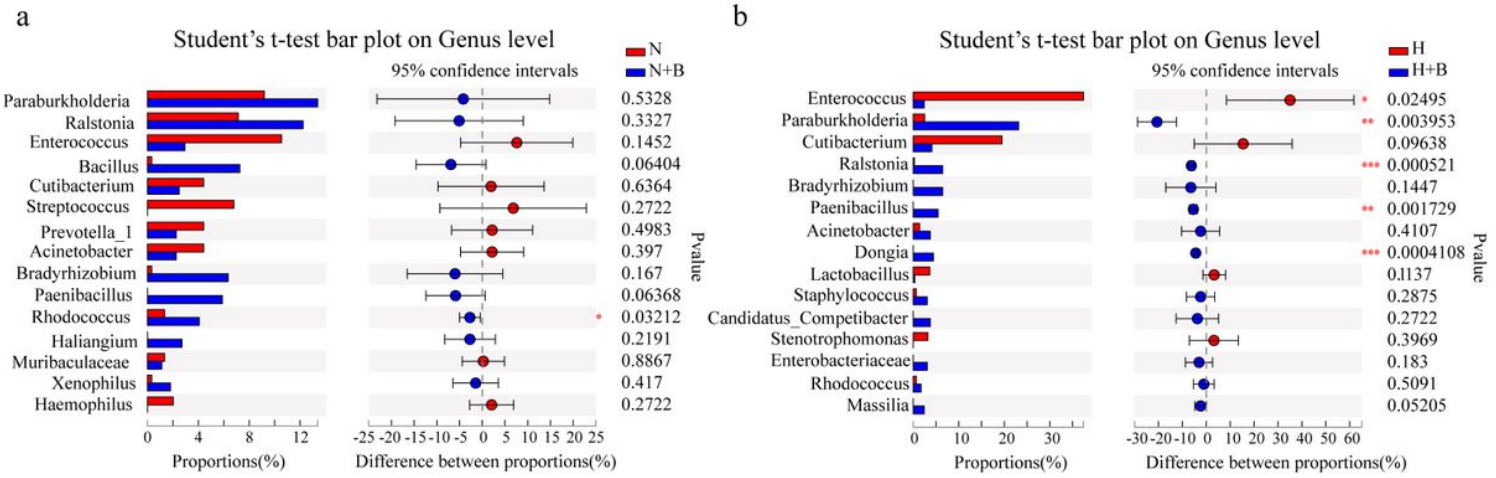

Figure 6

Differential abundance of bacterial genus levels in the root tissues of the inoculated and non-inoculated groups at normal and high temperatures. a Normal temperature. b High temperature. Only the richest 15 genera are depicted. Student's t-tests followed by Bonferroni ($P < 0.05$) corrections were performed at the genus level. * indicates a significant difference (Student's t-test, $P < 0.05$). ** indicates an extremely significant difference (Student's t-test, $P < 0.01$). *** indicates an extremely significant difference (Student's t-test, $P < 0.001$).

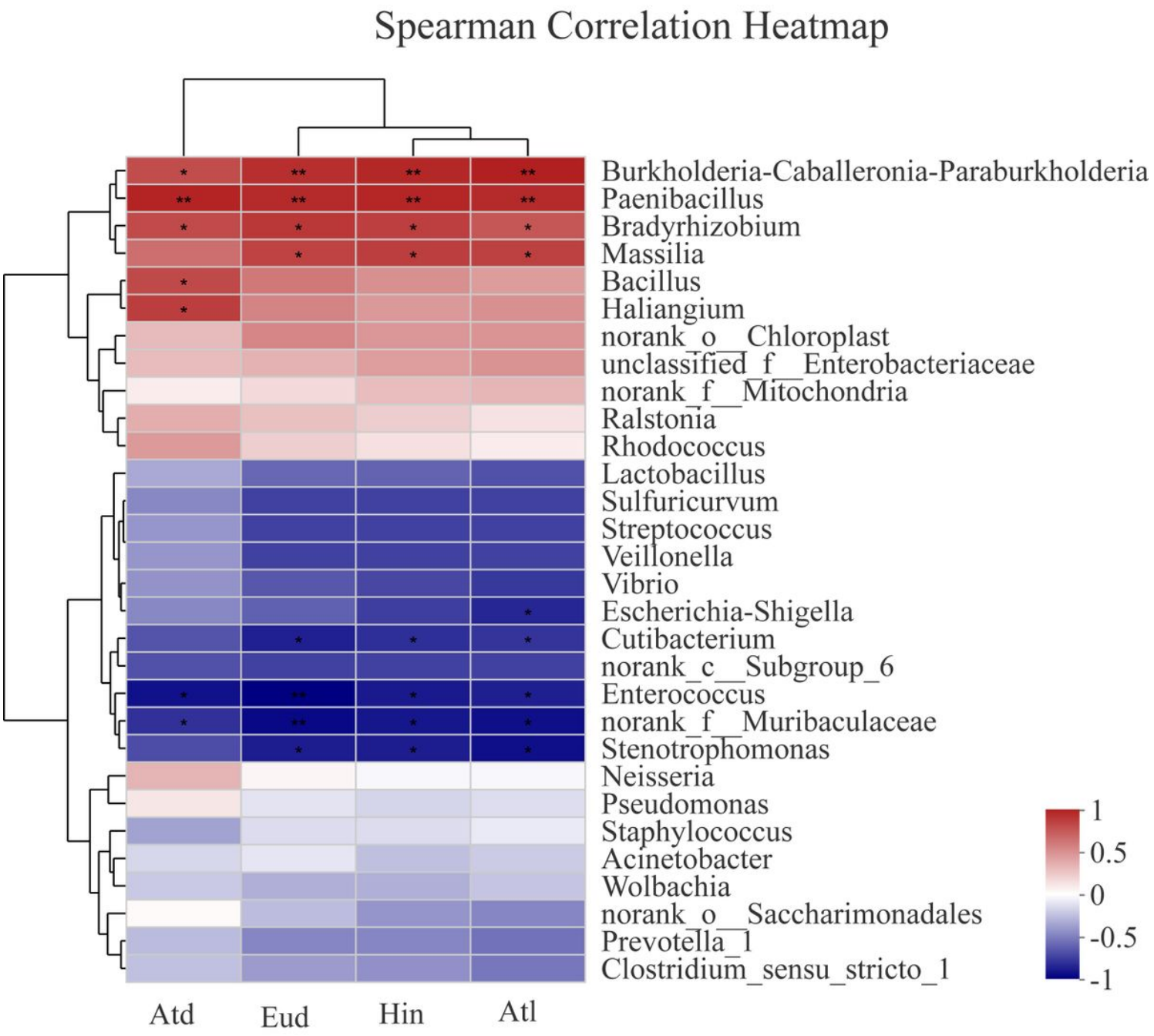

Figure 7

Spearman correlation analysis of four volatile oils of Atractylodes lancea and root endophytic bacteria at genus level. hinesol, hinesol; β-eudesmol, β-eudesmol; atractylon, atractylon; atractylodin, atractylodin. * indicates $0.01 < P \leq 0.05$, and ** indicates $0.001 < P \leq 0.01$.

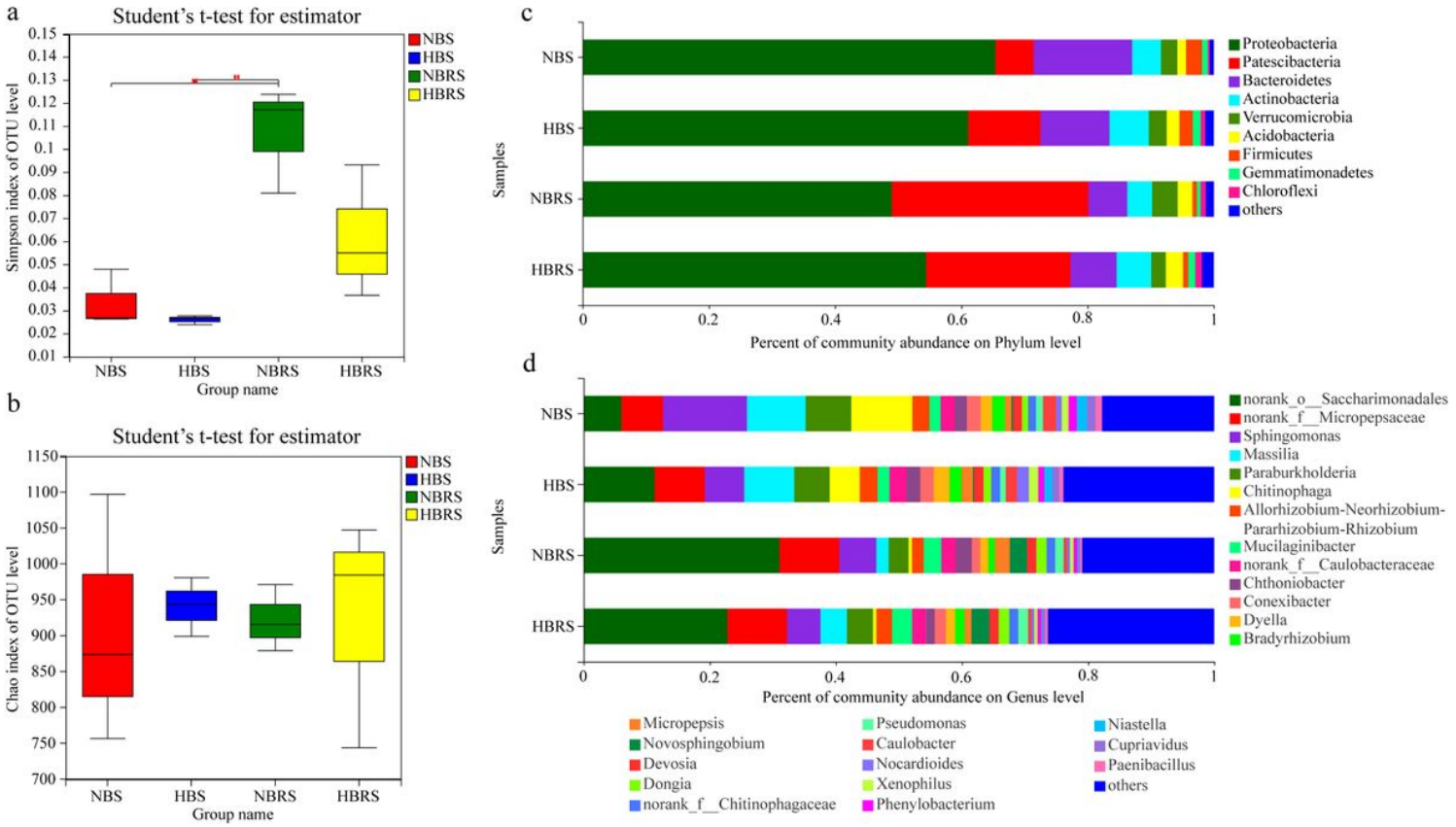

Figure 8

Comparative analysis of the alpha diversity and taxonomic distribution of 16S rRNA rhizosphere sequences from the rhizosphere soil of Atractylodes lancea plantlets in the inoculated and non-inoculated groups at normal or high temperature. a Shannon species diversity index. b ACE species richness index. c Phyla-level distributions in the rhizosphere samples based on 16S amplicon sequencing at normal or high temperature. d Genus-level distributions in the rhizosphere samples based on 16S amplicon sequencing at normal or high temperature. * indicates a significant difference (Student's t-test, P < 0.05).

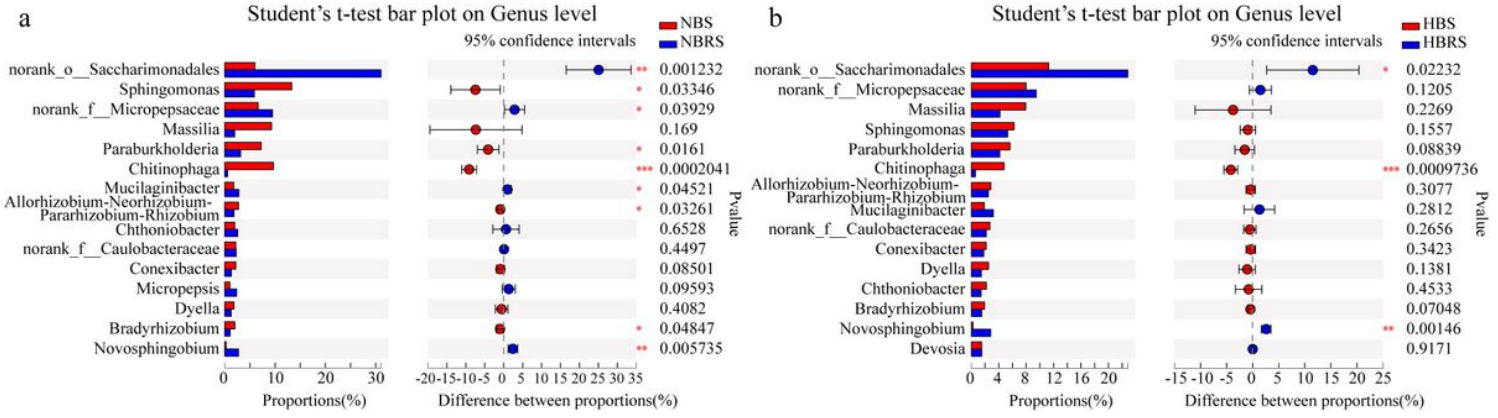

Figure 9

Differential abundance of bacterial genus levels in the control and rhizosphere soil of the inoculated and non-inoculated groups at normal or high temperature. a Normal temperature. b High temperature. Only

the richest 15 genera are depicted. Student's t-tests followed by Bonferroni ($P < 0.05$) corrections were performed at the genus level. * indicates a significant difference (Student's t-test, $P < 0.05$). ** indicates an extremely significant difference (Student's t-test, $P < 0.01$). *** indicates an extremely significant difference (Student's t-test, $P < 0.001$).

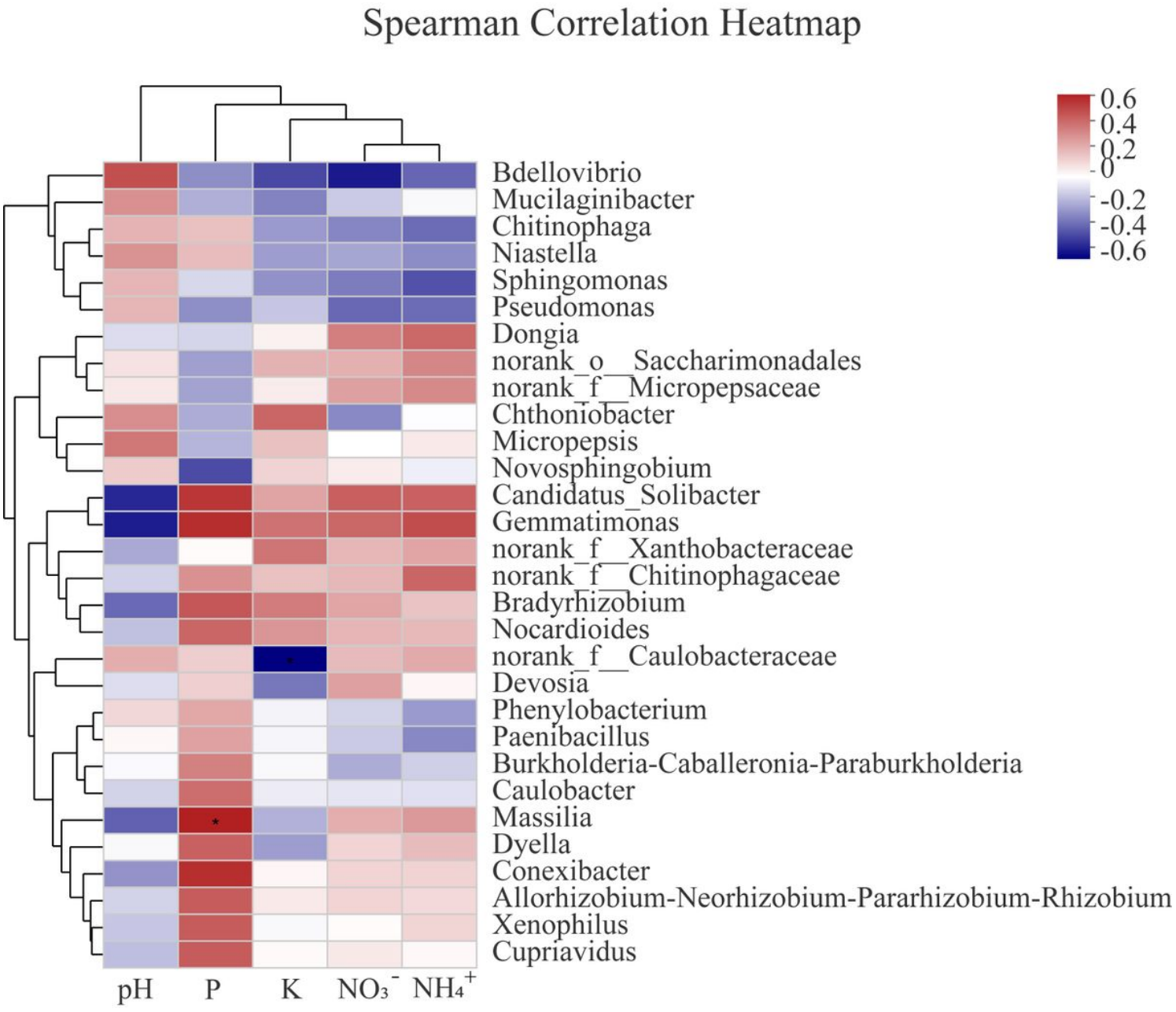

Figure 10

Spearman correlation analysis of soil chemical properties and rhizosphere bacteria at the genus level. pH indicates the pH value. P denotes available P. K stands for available K. NO3− denotes NO3−-N. NH4+ represents NH4+-N. * means $0.01 < P \leq 0.05$, and ** means $0.001 < P \leq 0.01$.

Supplementary Files

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