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Bacterial communities that metabolize 4-Hydroxybenzoic acid in grape (*Vitis vinifera* L.) rhizosphere soil

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ABSTRACT

The 4-Hydroxybenzoic acid (4-HBA) is one of the major autotoxins in grapevine soil. This study aimed to identify the bacteria involved in the metabolism of 4-HBA in grapevine rhizosphere soil. Grapevine rhizosphere soil was collected and treated at an interval of 48 h with 4-HBA (unlabelled or ^{13}C labelled) doses of 0, 5, 50 and 500 $\mu g \cdot g^{-1}$ dry soil. DNA-stable isotope probing combined with high-throughput sequencing was used to identify the microorganisms responsible for 4-HBA metabolism in the soil. In all, 12 phylotypes were found to utilize 4-HBA. Besides the 4-types of bacteria (*Pseudomonas, Cupriavidus*, *Azotobacter, Arthrobacte*) previously reported to be involved in the metabolism of 4-HBA, 8-others were also found in 4-HBA metabolism for the first time. These included *Arthrobacter*, *Pseudomonas*, *Methylophilaceae* uncultured, *Methylophilus*, *Legionella*, *Azoarcus*, *Azotobacter*, *Novosphingobium* and *Vogesella*

Key words: Allelopathy, autotoxin, bacteria, DNA-stable isotope probing, grapevine, 4-hydroxybenzoic acid, phytotypes, *Vitis vinifera*

INTRODUCTION

Grape (*Vitis vinifera* L.) is important fruit crop. However, due to its restricted cultivation conditions, it is affected by replant disease, which has become serious problem in old vineyards. Autotoxicity is intraspecific allelopathy that occurs when a plant species release chemical substances that inhibit or delay germination and growth of the same plant specie (1,36). It affects the growth of plants, reduce crop yields and causes replanting problems in agro-ecosystems (14,31,36). Phenolic compounds are putative allelochemicals (15) and studies have shown that autotoxic phenolic compound effects on target plants are mediated via soil microorganisms. For example, the poor performance of peanut plants grown in the soil treated with phenolic acids was attributed to the changes in soil microbial communities brought about by the phenolic acids (20,23). Significant associations between the crop (wheat, maize and soybean) growth and soil microbes have been reported after the application of chemicals [Veratric acid, maltol and (-)-loliolide (45)]. Replant disease of *Pseudostellaria heterophylla* is believed to be the result of increased proliferation of pathogenic *Fusarium oxysporum* stimulated by its root exudates, especially the phenolic compounds (44). A mixture of phenolic acids is believed to have mediated a shift in the structure of rhizosphere soil microflora of continuously monocultured *Radix pseudostellariae* L. and led to an increased replanting disease incidence (39). Thus,

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*Correspondence author, [1]Finance Department allelochemical- specific changes in the soil microbial community might adversely affect the plant growth. Therefore, identifying microorganisms that participate in the metabolism of allelochemicals is necessary to elucidate the interactions between the plants and soil microbes.

Our preliminary study (13) showed that 4-HBA is the autotoxin of grapevine (*Vitis vinifera* L.). Exogenously applied 4-HBA did not accumulate in the soil and the residual acid did not reach toxicity lever even after applying for 21d (22). Other than adsorption on soil particles, this chemical was apparently utilized by microorganisms, thereby, causing a disruption in soil microbial community balance by modifying the microbial population and community structure (12). The changing microbial communities stimulated by 4-HBA may cause autotoxicity and replant disease in the grapevine. In our previous study (12), the soil microbial community structure was evaluated by DGGE (denaturing gradient gel electrophoresis) method, which detected only those microorganisms that are numerically most abundant. The predominant microorganisms that are involved in the metabolism of 4-HBA in complex soil environments are unknown.

DNA-stable isotope probing (SIP) overcomes the limitations of traditional culturing and environmental molecular methods to analyse microorganisms (29,34). Microbial communities that actively utilize a wide variety of chemical compounds such as glucose, phenol, caffeine (32), benzene (41) and pentachlorophenol (21) have been detected *in situ* by using DNA-SIP method**.** This study aimed to determine the microbial groups that may be in smaller numbers but can metabolize 4-HBA in grapevine soil by using DNA-SIP combined with high-throughput sequencing.

MATERIALS AND METHODS

Material preparation and site description

The vineyard was established in 2004 in Shenyang Agricultural University, Shenyang City, Liaoning Province, China (41.8N°, 123.4°E; mean height above sea level, 82.6 m; annual average rainfall, 714 mm; annual average temperature, 6.2-9.7°C). The soil used was collected from another vineyard (established in 1978) from our University. It was renewed twice in 1990 and 2010 and the soil had remained fallow since 2010. The soil pH 6.67, was aquic brown and contained 101.03 $mg \cdot kg^{-1}$ available N, 96.54 $mg \cdot kg^{-1}$ available P, 128.1 $mg \cdot kg^{-1}$ available K, 31.83 $g \cdot kg^{-1}$ soil organic matter and 15% soil water content. The soil also contained 0.32 ± 0.08 $\mu g \cdot g^{-1}$ dry soil 4-HBA. Available N was determined using the alkali solution-diffusion method; available P by molybdenum blue colorimetric method; available potassium by flame photometric method and organic matter in soil mixture by the potassium dichromate method (3). Soil water content was determined by drying the powdered mixture at 105°C for 24 h in an oven. Soil pH was determined by using a soil suspension of 10 g of soil soaked in 50 mL water and shaken for 1 h.

Unlabelled and labelled 4-HBA (benzene ring-^{13}C, 99%) were purchased from J&K Scientific Ltd. (China) and Cambridge Isotope Laboratories (Andover, MA), respectively.

Plant growth conditions and rhizosphere soil collection

In all, 300 *V. vinifera* L. cv. Beta (*Vitis riparia* × *Vitis labrusca*) cuttings (root promoted in an electric thermal bed) were planted in plastic pots (8.5 × 5 × 20 cms) containing 1500 g soil (soil from the above vineyard + peat moss + sand in a ratio of

7:2:0.5 v/v). There was one cutting per pot. All cuttings were maintained under rain-shelter (28 °C day/18 °C night, 16 h light/8 h dark) between May 1 and July 31, 2014.

At 60 days after planting, 100 plants were gently uprooted, large blocks of soil adhering to the root was shaken off and only the soil adhering to the roots (0-4 mm) was collected by gentle brushing and this was used as rhizosphere soil (24). By this 7, 200 g (dry weight) rhizosphere soil was collected from 100 plants. The soil was pooled, sieved through a 2-mm sieve and thoroughly homogenised by hand and transported to the laboratory in airtight coolers and stored at 4°C until further use.

Application of 4-HBA to the rhizosphere soil

The experiment was conducted from August 1 to 15, 2014. 4-HBA was dissolved in 100% methanol and then diluted with sterile double-distilled water (2:98, v/v) to obtain concentrations of 8.5, 85 and 850 μg·mL^{-1}. Then methanol was added into low concentrations to maintain the same content of methanol in all treatments. Because soil pH is key factor that regulates soil microbial communities (11), the pH of the solution was adjusted to 7.0 by using 0.1 M NaOH.

The rhizosphere soil (85 g dry weight) was added in each pot (8.5× 6× 6 cms). Rhizosphere soil water content was adjusted to about 60% of maximum water holding capacity by using sterile double-distilled water. Subsequently, 10 mL of 8.5, 85 and 850 μg·mL^{-1} unlabelled and labelled 4-HBA solution (at 48 h interval) was applied to the rhizosphere soil 5-times. In control 10 mL methanol diluted with sterile double-distilled water as treatments was applied into the rhizosphere soil. The final dose was 0, 5, 50 and 500 μg·g^{-1} dry soil, respectively. (The 0 represent the dose of 4-HBA that we added to the soil and not the actual dose of 4-HBA in the soil). These doses of 4-HBA were based on the actual dose detected in the grapevine rhizosphere soil in Shenyang Agricultural University vineyard (established in 2004) (unpublished). The pots were then randomly placed in growth chamber (Panasonic, Japan) with a photoperiod of 16 h light (80% relative humidity) and 8 h dark (60%) at 25°C. Twelve days after the first treatment with 4-HBA (including unlabelled and labelled 4-HBA), the soil was sampled. Every treatment had 9 replicates. Three soil replicates were obtained by pooling the three sub-replicates into one sample for further analysis to reduce the variability between the replicates within the same treatment (2). Three replicates were randomly chosen and mixed into one sample. Thus 9 replicates turned into 3 for each treatment. Ten g soil from each replication was sampled and used for DNA extraction. The soil samples were stored at -20°C for soil DNA extraction.

Soil DNA extraction and SIP gradient fraction

Total DNA in soil samples was extracted by using a Fast DNA SPIN kit for soil (MP Biochemicals, Solon, OH, USA) following the manufacturer's instructions. Soil DNA was dissolved in 100 μL nuclease-free water. The quantity and purity of DNA were determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

DNA-SIP was performed using isopycnic density centrifugation as previously described (40). Briefly, 3 μg of total soil DNA extract was mixed with an initial 4.9 mL CsCl solution of buoyant density of 1.725 g·mL^{-1} in 5.1-mL Beckman polyallomer ultracentrifuge tubes and centrifuged at 177, 000 × *g* for 44 h at 20°C in a Vti65.2 vertical rotor (Beckman Coulter, Inc., Palo Alto, CA, USA). Gradient medium with sterile water was displaced from the top of the ultracentrifuge tubes by using an NE-1000 single syringe pump (Baoding Longer Precision Pump Co., Ltd, China). Fifteen DNA fractions (380 μL) were obtained for each sample and 60-μL aliquots of each fraction were used to determine the refractive index by using an AR200 digital hand-held refractometer (Reichert, Inc., Buffalo, NY). The DNA was separated from the CsCl solution by adding 2 volumes of polyethylene glycol 6000 (PEG 6000) in 1.6 M NaCl at 37°C for 1 h, followed by centrifugation at 13,000× *g* for 30 min. The fractionated DNA was further purified with 70% ethanol and dissolved in 30 μL Tris-EDTA (TE) buffer.

PCR amplification

The 515F (5′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTT TRA GTTT-3′) primers (Shanghai Majorbio Bio-Pharm Technology Co., Ltd) were used to amplify the 16S rRNA gene amplicons; the primers targeted the V4-V5 regions (42). The PCR amplifications were performed in triplicate for each sample including total genomic DNA sample from control and labelled samples. PCR amplification used 20-μL reaction mixture containing 0.8 μL of each primer (5 μM), 2 μL of 2.5 mM deoxynucleoside triphosphate, 4 μL of 5X Fast *Pfu* buffer, 0.4 μL of Fast *Pfu* DNA polymerase and 10 ng of template DNA. PCR conditions were as follows: 95°C for 2 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 5 min.

High-throughput amplicon sequencing

After the PCR amplification, samples from labelled and unlabelled treatment, each containing three heavy fractions (V, VI, VII) and total genomic DNA sample from labelled samples and control were sequenced. Agarose gel (2%) electrophoresis was used to extract the PCR products. The products of interest were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, US) as per the manufacturer's instructions and quantified using QuantiFluor™-ST (Promega, US). Purified amplicons were pooled in equimolar concentrations and paired-end sequenced (2 × 250) on an Illumina MiSeq PE250 platform according to the standard protocols at the Shanghai Majorbio Bio-Pharm Technology Co., Ltd.

The analysis selected three heavy fractions (V, VI, VII) of the fifteen DNA fractions from the three labelled and unlabelled treatments, respectively. The total DNA samples of the control, labelled-5, labelled-50 and labelled-500 were also analysed using high-throughput sequencing to characterise the total microbial community present in these samples. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database under the accession ID SRP114955.

Sequence analysis and phylogenetic classification

Paired-end reads from the original DNA fragments were merged using FLASH (v.1.2.7, http://ccb.jhu.edu/softw are/FLASH/) (25) and assigned to each sample according to the unique barcodes. Samples from 1 labelled and 1 unlabelled treatment each containing 3 fractions and 1 total genomic DNA sample and control (thus, a total of 22 samples) were analyzed together. Raw fastq files were demultiplexed and quality-filtered using QIIME (v.1.17) with the following criteria: (i) The 250 bp reads were truncated at any site receiving an average quality score <20 over a 10 bp sliding window, discarding the truncated reads that were shorter than 50bp. (ii) exact barcode matching, 2 nucleotide mismatch in primer matching, reads containing ambiguous characters were removed. (iii) only sequences that overlap longer than 10 bp were assembled according to their overlap sequence. Reads which could not be assembled were discarded. Operational Units (OTUs) were clustered with 97% similarity cutoff using UPARSE (v.7.1 http://drive5.com/uparse/) (9) and chimeric sequences were identified and removed using UCHIME. The OTUs were then grouped into taxonomical levels by the RDP classifier (version 2.2, http://sourceforge.net/projects/rdp-classifier/) (37) at the order level within each bin at 97 % similarity cut off level. Rare faction curves based on Mothur v.1.30.1 are shown in Supplementary Figure 1. The sequencing results for the different doses of labelled 4-HBA treatment are shown in Supplementary Table 1.

Statistical Analysis of Data: Heatmap was prepared using the data of relative abundance of each bacterial genus in four samples (total DNA in 0, 5, 50, 500 μg·g-1 4-HBA-amended soil) with the R software package. Variables were clustered on the vertical axis. The phylogenetic tree was calculated using the approximate-maximum-likelihood and relationships among samples were determined with Bray distance and complete method.

Hierarchical clustering analysis (HCA) was done using the data of relative sequence abundance (percent of sequences detected for each OUT at 97% level for each sample) on OUT level. Hierarchical clustering (Average Neighbour, also known as UPGMA) was used to analyse community distance matrices to generate visual representations of multivariate community structure similarities among samples with the software Qiime (R package). The Beta diversity was used on data subsets within hierarchical clustering models to test whether communities differed significantly between labelled and unlabelled 4 HBA fractions.

RESULTS AND DISCUSSION

Microbial community analysis

Many studies have shown that 4-HBA could be utilized by microorganisms, when it is sole carbon source. Chen *et al.* (7) reported that *Pseudomonas putida* isolated from the cucumber rhizosphere soil degrades the 4-HBA. Some fungi such as *Phomopsis liquidambari* and *Trichoderma harzianum* also degrade the 4-HBA (6,8). In these studies, pure microbial strains were used to degrade the 4-HBA. However, to our knowledge, the present is the first *in situ* study to identify the specific bacteria that metabolize the 4-HBA.

In our experiment, the sequence analysis of total genomic DNA samples from four treatments indicated that the bacterial community consisted of 10 major phyla. Of all the sequences identified, *Proteobacteria* was the most abundant phyla in all the four treatments (all, >80%). *Bacteria_ unclassified* was 1.1%-3.5%. The other phyla were [*Actinobacteria* (1.7%-4.4%), *Bacteroidetes* (1.4%-3.1%), *Chloroflexi* (1.2%-2.6%), *Acidobacteria* (0.9%-1.3%), *Gemmatimonadetes* (0.3%-1.1%) and *Verrucomicrobia* (0.4%-1.2%)]. *Firmicutes*, *Nitrospirae* and *Planctomycetes* were also present in these four micrococosoms in range of 0.1%-0.7%. The relative abundance of microorganisms belonging to *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Gemmatimonadetes, Verrucomicrobia* and *Nitrospirae*, in 0 μg·g^{-1} 4-HBA-treated soil was lower than in soil treated with the labelled 4-HBA (Fig. 1).

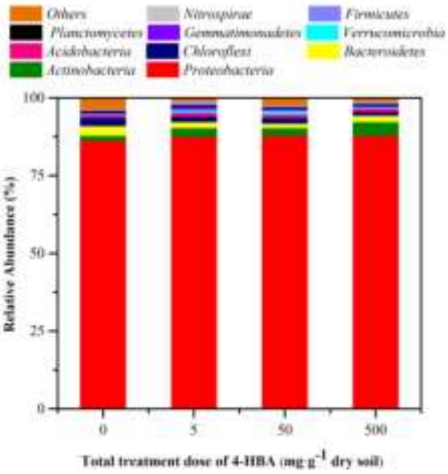

Figure 1. The relative abundance of total genomic DNA sequences extracted from soil samples amended with ^{13}C labelled 4-HBA at the phylum level.
The relative abundance under 0.3 % was classified into others.

The heatmap plot prepared based on the abundant bacterial community at the genus level showed that *Methylobacillus*, *Methylophilaceae_uncultured*, *Methylophilus* and *Methylophilaceae _unclassified* were the dominant genera in all treatments (Fig. 2).

The heatmap plot depicts the relative abundance of each bacterial genus within each sample. The relative values for bacterial genera are indicated by colour intensity in the legend. The relative abundance under 0.3 % was classified into others.

Phylotype relative abundance across SIP (Stable isotope probing) fractions

In this study, SIP was applied to identify the 4-HBA decomposing bacteria by adding the ^{13}C-4HBA in soil samples. This approach can provide information about which microbial populations are present and which members are degrading the ^{13}C-4-HBA. Total DNA was extracted from the soil and the ^{13}C-enriched DNA from cells that added the ^{13}C derived from labeled 4-HBA, was separated from unlabelled DNA, by ultracentrifugation. After density-gradient centrifugation, the DNA in each fraction in ^{12}C 4-HBA and ^{13}C 4-HBA amended soil were different. Comparing the relative abundance of these phylotypes in same heavy fraction between ^{12}C DNA and ^{13}C DNA, their relative abundance was more in ^{13}C DNA than in ^{12}C DNA, would be considered as involving in metabolism of 4-HBA in grapevine rhizosphere soil (17).

Hierarchical cluster analysis showed that three groups were apparent (i.e. a_7; a_5 and a_6; A_5, A_6 and A_7) in 5 µg·g^{-1} 4-HBA amended soil (Fig. 3A). Similarly, three groups were clustered (i.e. b_7; b_5 and b_6; B_5, B_6 and B_7) in 50 µg·g^{-1} 4-HBA amended soil (Figure 3B). Two groups were clustered (i.e. c_5, c_6 and c_7, C_5, C_6 and C_7) in 500 µg·g^{-1} 4-HBA amended soil (Figure 3C). Apparently different buoyant fractions were grouped together in labelled 4-HBA amended soil. Labelled and unlabelled 4-HBA treatments were clearly separated with the increasing 4-HBA concentration.

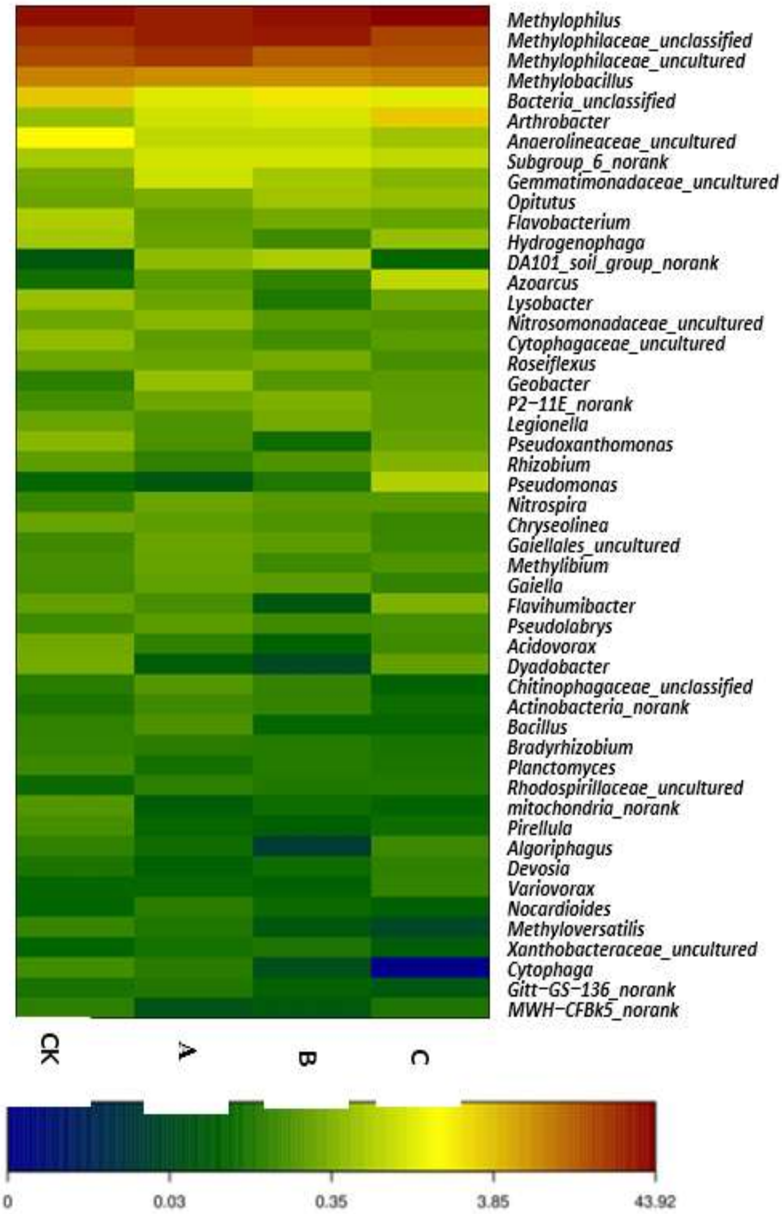

Figure 2. Bacterial distribution of the top 50 abundant genera among four samples.
CK, A, B and C represent labelled 0, 5, 50 and 500µg·g^{-1} 4-HBA-amended soil, respectively.

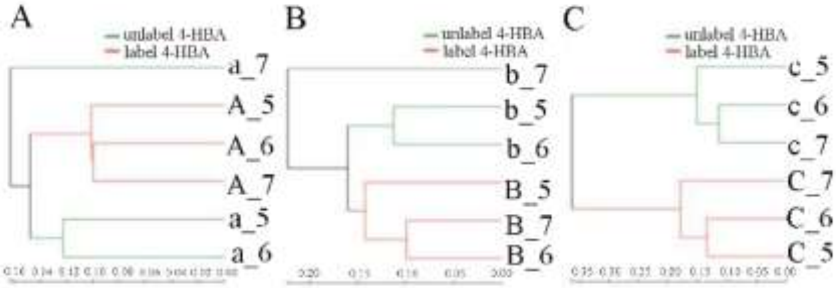

Figure 3. Hierarchical cluster analysis (HCA) on OUT levelin4-HBA amended soils
(A) 5μg·g^{-1} 4-HBA-amended soil, (B) 50μg·g^{-1} 4-HBA-amended soil, (C) 500μg·g^{-1} 4-HBA-amended soil.
Capital letters (A, B, C) indicate the soil was treated with labelled 4-HBA, lowercase letters (a, b, c) indicated the soil was treated with unlabelled 4-HBA. Arabic numerals (5, 6, 7) indicated buoyant density fraction from heavy to light.

The ^{12}C substrate and ^{13}C substrate have similar physicochemical properties, except different mass Numbers. Growth of soil microbes in ^{13}C labelled substrate increased the density of DNA than from soil microbes grown in ^{12}C labelled substrate. According to the different densities of ^{12}C DNA and ^{13}C DNA, ^{13}C labelled and unlabeled DNA of soil microbes could be separated by density-gradient centrifugation (34). After centrifugation, the relative abundance of these phylotypes in heavy fractions was compared between the labelled and unlabelled 4-HBA-amended microcosms. The phylotypes responsible for the uptake of labelled 4-HBA were determined by analysing the heavy fractions with higher DNA concentration in the labelled 4-HBA-amended soil than in the unlabelled 4-HBA-amended soil. The Supplementary Fig 2 is the DNA concentration in labelled 4-HBA and unlabelled 4-HBA soil.

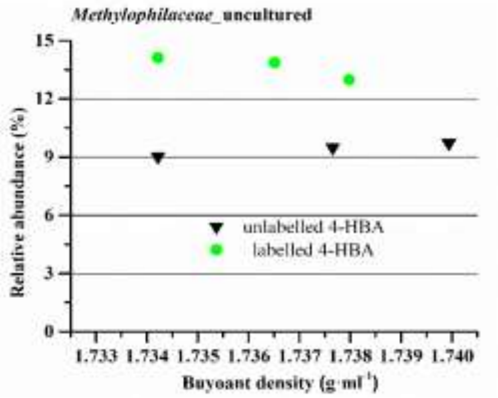

Figure 4. The relative abundance of active phylotypes in heavy fractions in soil from labelled and unlabelled 4HB
The relative abundance under 0.3 % was classified into others. Amendment at s5 μg·g^{-1} 4-HBA

The relative abundance of family *Methylophilaceae*_uncultured (*Proteobacteria*) was higher in 5 μg·g^{-1} labelled 4-HBA-amended soil than in 5 μg·g^{-1} unlabelled 4-HBA-amended soil (Fig. 4).

Bacteria from the genera *Methylophilus* (*Proteobacteria*), *Legionella* (*Proteobacteria*) and *Blastococcus* (*Actinobacteria*) had higher relative abundance in 50 μg·g^{-1} labelled 4-HBA-amended soil than in 50 μg·g^{-1} unlabelled 4-HBA-amended soil (Figure 5). Further, the relative abundance of *Actinobacteria*, including *Arthrobacter* and *Blastococcus* and that of *Proteobacteria*, including *Azoarcus*, *Novosphingobium*, *Pseudomonas*, *Cupriavidus*, *Vogesella*, *Ramlibacter* and *Azotobacter*, were higher in 500 μg·g^{-1} labelled 4-HBA-amended soil than in 500 μg·g^{-1} unlabelled 4-HBA-amended soil (Figure 6). The phylotypes identified in present study were from the phyla *Proteobacteria* and *Actinobacteria*.

In this study, the type and diversity of microorganisms involved in 4-HBA metabolism in grapevine soil depended on the 4-HBA concentration. With increasing 4-HBA concentration, different number of phylotypes were detected. The phylotypes identified in this study were from the phyla *Proteobacteria* and *Actinobacteria*. Studies have shown that *Proteobacteria* had the ability to degrade phenolic compounds, including 4-HBA and catechol (26,33). Further, *Actinobacteria* degrades the phenolic compounds (27). Our findings confirm that *Proteobacteria* and *Actinobacteria* were involved in the metabolism of 4-HBA in grapevine rhizosphere soil.

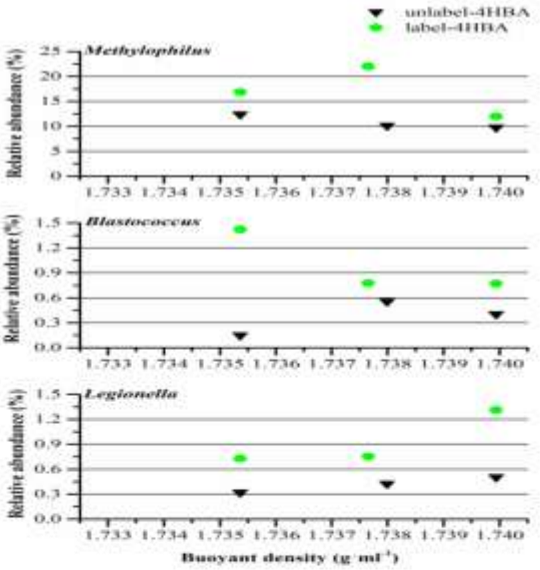

Figure 5. The relative abundance of active phylotypes in heavy fractions from soils amended with labelled and unlabelled 4-HBA
The relative abundance under 0.3 % was classified into others. Amendment at 50 μg·g^{-1} 4-HBA

Several phylotypes were more abundant in heavy fractions from the ^{13}C-labeled 4-HBA-amended soil microcosms compared to heavy fractions from the unlabeled 4-HBA soil. The relative abundance of 12 phylotypes was higher in labelled 4-HBA-amended soil than in unlabelled 4-HBA-amended soil, which were involved in metabolism of 4-HBA. Four (*Pseudomonas, Cupriavidus*, *Azotobacter, Arthrobacter*) of the 12 phylotypes have been reported to be involved in metabolish of 4-HBA, however the work concerned only with 4-HBA metabolic pathway using it as the sole carbon and energy source, and pure microbial strains were used to degrade 4-HBA. Our experiment explored the 4-HBA metabolism *in situ.* Besides the four types of bacteria previously reported, we identified other eight to participate in 4-HBA metabolism for the first time under situ condition. Of these phylotypes, *Arthrobacter*, *Pseudomonas*, *Methylophilaceae*_uncultured, *Methylophilus*, *Legionella*, *Azoarcus, Azotobacter*, *Novosphingobium*, *Vogesella* reported earlier are also involved in N fixation or denitrification (4,5,10,16,18,19,28,30,38,43). Thus utilization of 4-HBA may only be an addition property of this group of bacteria.

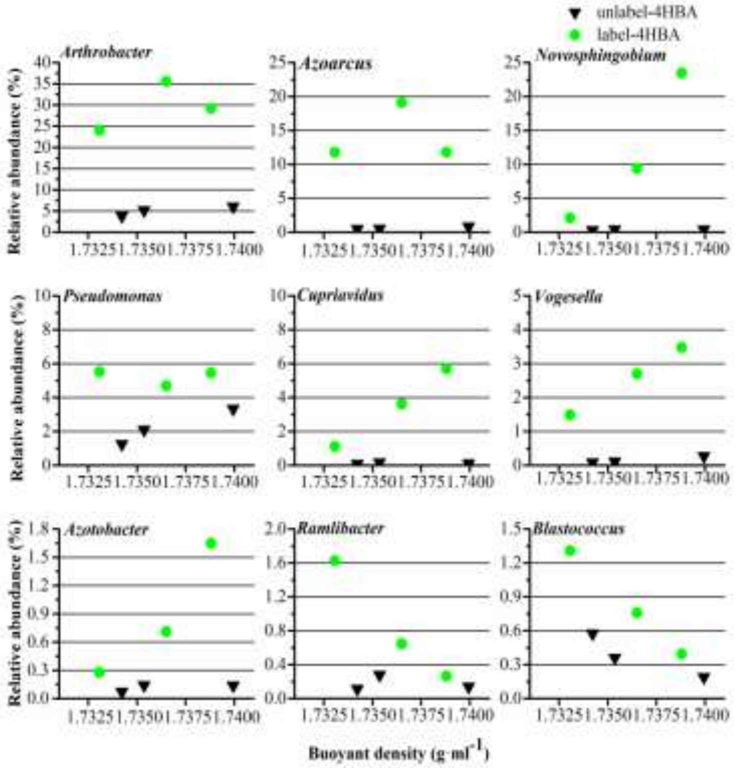

Figure 6. Relative abundance of active phylotypes in heavy fractions from soils amended with labelled and unlabelled 4-HBA
The relative abundance under 0.3 % was classified into others. Amendment was at 500 $\mu g \cdot g^{-1}$ 4-HBA

SIP-DNA is useful to identify the microorganisms involved in the metabolism of specific substrates (32,34,35). In this study, microorganisms responsible for 4-HBA metabolism in the soil were not the dominant genera that were observed in all 4-HBA-treated soils. This indicated that the efficacy of SIP approach. However, determining whether the microbes that participated in 4-HBA metabolish assimilated 4-HBA, as primary utilizers or used the transferred products by secondary utilizers was difficult to conclude. The phylotypes determined in this study might have resulted from the cross-feeding of sub-products of 4-HBA consumption by other bacteria. In this study, a universal primer was used to amplify the 16S rRNA genes and determine the function of microorganisms as the group involved in 4-HBA metabolism was not known. The study provides new insight into the relationships between 4-HBA and soil bacteria.

CONCLUSIONS

We found that the major autotoxins were secreted by the root system of grape, however, 4-HBA can be utilized by microorganisms. For the first time under situ condition, we found that 12-phylotypes utilised the 4-HBA. Besides the 4-types of bacteria (*Pseudomonas, Cupriavidus*, *Azotobacter, Arthrobacte*) involved in the metabolism of 4-HBA, 8-other bacteria (*Arthrobacter*, *Pseudomonas*, *Methylophilaceae* uncultured, *Methylophilus*, *Legionella*, *Azoarcus*, *Azotobacter*, *Novosphingobium* and *Vogesella)* also participated in the 4-HBA metabolism.

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