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RESEARCH ARTICLE

α-Tomatine gradient across artificial roots recreates the recruitment of tomato root-associated *Sphingobium*

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Abstract

α-Tomatine is a major saponin that accumulates in tomatoes (*Solanum lycopersicum*). We previously reported that α-tomatine secreted from tomato roots modulates root-associated bacterial communities, particularly by enriching the abundance of *Sphingobium* belonging to the family *Sphingomonadaceae*. To further characterize the α-tomatine-mediated interactions between tomato plants and soil bacterial microbiota, we first cultivated tomato plants in pots containing different microbial inoculants originating from three field soils. Four bacterial genera, namely, *Sphingobium*, *Bradyrhizobium*, *Cupriavidus*, and *Rhizobacter*, were found to be commonly enriched in tomato root-associated bacterial communities. We constructed a pseudo-rhizosphere system using a mullite ceramic tube as an artificial root to investigate the influence of α-tomatine in modifying bacterial communities. The addition of α-tomatine from the artificial root resulted in the formation of a concentration gradient of α-tomatine that mimicked the tomato rhizosphere, and distinctive bacterial communities were observed in the soil close to the artificial root. *Sphingobium* was enriched according to the α-tomatine concentration gradient, whereas *Bradyrhizobium*, *Cupriavidus*, and *Rhizobacter* were not enriched in α-tomatine-treated soil. The tomato root-associated bacterial communities were similar to the soil bacterial communities in the vicinity of artificial root-secreting exudates; however, hierarchical cluster analysis revealed a distinction between root-associated and pseudo-rhizosphere bacterial communities. These results suggest that the pseudo-rhizosphere device at least partially creates a rhizosphere environment in which α-tomatine enhances the abundance of *Sphingobium* in the vicinity of the root. Enrichment of *Sphingobium* in the tomato rhizosphere was also apparent in publicly available microbiota data, further supporting the tight association between tomato roots and *Sphingobium* mediated by α-tomatine.

KEYWORDS
α-tomatine, bacterial communities, pseudo-rhizosphere device, rhizosphere, *Sphingobium*, tomato

1 | INTRODUCTION

The rhizosphere, defined as soil area under the influence of plant roots (Hartmann et al., 2008), harbors microbial communities that exert various effects on plant growth and physiology (Bakker et al., 2018; Vacheron et al., 2013). A large amount of plant metabolites are secreted from the roots to soil and shape the microbial communities around the plant roots, forming root-associated microbiota (Hong et al., 2022; Sasse et al., 2018). Plant-specialized metabolites (PSMs) are predominantly attributable to differences in root-associated microbiota, whose characteristics depend on plant species and are altered according to the growth conditions and stresses (Korenblum et al., 2020; Ma et al., 2022). In addition, root phenotypic traits and architecture also influence microbiome assembly (Robertson-Albertyn et al., 2017; Saleem et al., 2018). Root-associated microbiota formed by PSMs can be beneficial to plant growth (Huang et al., 2019; Korenblum et al., 2022); for example, coumarin secreted under iron deficiency modifies root-associated microbiota to improve plant health and immunity in *Arabidopsis thaliana* (Harbort et al., 2020; Stringlis et al., 2018; Voges et al., 2019); benzoxazinoids secreted from maize roots alter microbial communities, improving plant growth and herbivore defense (Cotton et al., 2019; Hu et al., 2018); and flavones secreted from maize roots can enrich *Oxalobacteraceae*, improving the nutrient uptake of maize under nitrogen deficiency (Yu et al., 2021). These studies used plant mutants that were defective in PSM biosynthesis and compared the root-associated microbiota between mutant and wild-type plants. Although the analyses of plant mutants revealed the involvement of PSMs in shaping root-associated microbiota, there remains a possibility that other metabolites in these mutants affect soil microbes. To circumvent the effects of metabolites in root exudates other than the PSMs of interest, direct application of PSMs to the soil has been employed (Maver et al., 2021; Okutani et al., 2020; Schutz et al., 2021). The application of daidzein to field soil altered bacterial communities to resemble those in the soybean rhizosphere, typically by enriching *Comamonadaceae* (Okutani et al., 2020). Other PSMs, such as saponins and alkaloids, also modify the soil microbiota when added to the soil (Fujimatsu et al., 2020; Maver et al., 2021; Nakayasu, Yamazaki, et al., 2021; Shimasaki et al., 2021; Sugiyama, 2021).

Although the application of PSMs to the soil is a favorable method to demonstrate the roles of PSMs in modifying the soil microbiota, especially when it is difficult to obtain mutants, the concentration gradient of PSMs in the rhizosphere cannot be reproduced (Sugiyama, 2023). To establish the concentration gradient of PSMs in direct application methods, rhizobox and artificial root systems have been employed in combination with rhizosphere models. Rhizon samplers were employed to collect various components in the rhizosphere including soil water, micro elements, dissolved organic carbon, exudates, and enzymes (Dobermann et al., 1994; Farley & Fitter, 1999). In addition, experiments using rhizobox were conducted to analyze the distribution of metabolites in soil near roots (Helal & Sauerbeck, 1983; Youssef & Chino, 1988). These experimental setups are also utilized to evaluate the spatial distribution and functional roles of metabolite in the rhizosphere. Szoboszlay et al. (2016) demonstrated that the treatment of 7,4′-dihydroxyflavone from Rhizon samplers as an artificial root into the soil modified bacterial communities, but naringenin did not cause the siginificant alternation of the soil bacterial community structure (Szoboszlay et al., 2016). Zhang et al. (2019) showed that the application of primary metabolites, such as glucose, alanine, methionine, citric acid, and malic acid, using Rhizon samplers as an artificial root induced the activities of microbial enzymes, such as phosphatase and sulfatase, in the vicinity of artificial roots (Zhang et al., 2019). Buckley et al. (2022) analyzed the effect of continuous sucrose supply on soil inorganic nitrogen flux using a microdialysis membrane as an artificial root and revealed that sucrose exudation induced a significant reduction in N fluxes, which did not recover after ceasing sucrose release (Buckley et al., 2022). Daidzein analyzed in rhizobox and modeling revealed the limited daidzein distribution within 2 mm from the root surface (Okutani et al., 2020; Toyofuku et al., 2021).

In both mutant analysis and PSM application studies, one type of soil is typically used as the source of microbes, although the soil microbiota is a dominant factor in influencing the formation of root-associated bacterial communities (Thiergart et al., 2020). Analyses of PSM-mediated interactions between plant and soil microbes comprising different community profiles would substantially contribute to our understanding of the roles of PSMs in the rhizosphere.

α-Tomatine, a major PSM of tomato accumulated in roots, leaves, and green fruits (Friedman, 2002), has been investigated for its toxicity for tomato pathogens and pests (You & van Kan, 2021). We have previously revealed the involvement of α-tomatine in shaping tomato root-associated bacterial communities (Nakayasu, Ohno, et al., 2021). Application of α-tomatine to the soil led to the enrichment of *Sphingomonadaceae*, which was also enriched in tomato root-associated bacterial communities. However, its relative abundance, in particular the genus *Sphingobium*, was reduced in root-associated bacterial communities of tomato jasmonate-responsive ETHYLENE RESPONSE FACTOR 4 (*jre4*) mutants, which accumulate and secrete a reduced amount of α-tomatine (Nakayasu, Ohno, et al., 2021). Our previous study revealed that α-tomatine enriched *Sphingobium* in shaping tomato root-associated bacterial communities. However, it remains elusive whether α-tomatine enriches *Sphingobium* when tomatoes are grown in different soils and whether it contributes to the enrichment of other root-associated bacteria.

In this study, we first investigated the root-associated bacterial communities of tomato plants cultivated with three different microbial inoculants to determine the common bacterial genera that are enriched in tomato roots and deepen our understanding of α-tomatine-mediated formation of tomato root-associated bacterial communities. We then constructed a pseudo-rhizosphere system to evaluate the formation of tomato root-associated bacterial communities mediated by α-tomatine.

2 | MATERIALS AND METHODS

2.1 | Chemicals

α-Tomatine samples were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) or Nacalai Tesque Inc. (Kyoto, Japan) unless otherwise stated.

2.2 | Soil collection and plant materials

Field soils were collected from three sites in Japan: a field in Kyoto University (Kyoto) (35.0298 N, 135.7873 E), a field in Tokyo University of Agriculture and Technology (Fuchu) (35.6840 N, 139.4786 E), and a field in Kyoto University of Advanced Sciences (Kameoka) (34.9938 N, 135.5514 E) (Figure S1). All fields have a previous history of tomato growth. Soil was air-dried and passed through a 2-mm sieve. Soil analysis was performed by the Tokachi Agricultural Cooperative Federation (Table S1). Tomato seeds (*Solanum lycopersicum* cv. Micro-Tom) were purchased from Inplanta Innovations Inc. (Kanagawa, Japan). Tomato seeds were soaked in 70% ethanol for 1 min and then in 5-times-diluted kitchen bleach (6% sodium hypochlorite, Kao Corporation) for 15 min for sterilization. Seeds were rinsed 5 times with sterile water, and nine seeds on average were sown on Murashige and Skoog (MS) medium agar in a plant box, which was placed in an incubation chamber (25°C, continuous light conditions).

2.3 | Tomato cultivation and sampling

Toyoura sand from Toyoura Silica Stone Mining Co., Ltd. (Yamaguchi, Japan) was sterilized via γ-radiation at an intensity of 30 kGy by the Japan Irradiation Service. Soil from each field was mixed with sterilized Toyoura sand at a volume ratio of 1:9. Autoclaved Agripots (BBJ Hitech Saitama, Japan; 135 ml) were filled with a mixture of soil and Toyoura sand. Four-week-old tomato seedlings were rinsed with sterile water to remove MS agar medium and were transplanted into the pots. As a nutrient source, 10 ml of autoclaved sterile tap water containing .02% (w/v) Otsuka House No. 1 and No. 2 standard nutrients (Otsuka Chemical Co., Osaka, Japan) was added to each pot. The mass water content was adjusted to 20% by adding sterile tap water. Autoclaved plant culture boxes (BBJ Hitech), which have the vents with cotton, were connected to the lower part of the Agripot containing the plants. Tomatoes were cultivated for 3 weeks at 25°C under continuous light conditions. Leaves were sampled, weighed, frozen in liquid nitrogen, and stored at −80°C. Roots were removed from the soil; suspended in phosphate-buffered saline containing 130-mM NaCl, 7-mM Na_2HPO_4, 3-mM NaH_2PO_4 (pH 7.0), and .02% Silwet L-77 (Nippon Unicar Co., Ltd., Kanagawa, Japan); and sonicated for 5 min. After rinsing with tap water, the water was removed by placing the plants on a paper towel, and fresh weights were estimated. Roots were frozen in liquid nitrogen and stored at −80°C until use. Bulk soils were sampled from the pots without tomato plants.

2.4 | Construction of a model experimental system and sampling

A mullite ceramic tube (inner diameter, 3 mm; outer diameter, 5 mm; length, 100 mm; 1DH3050, Sakaguchi Dennetsu K.K.) was used as an artificial root. The ceramic tube was sealed at the bottom with adhesive (Cemedine Co., Ltd., Tokyo, Japan), and a 200-µl tip was placed on the top. γ-Sterilized Toyoura sand was mixed with Kameoka field soil at a volume ratio of 19:1. Sterile tap water was added to obtain a water content of 20%. Sterile plastic cups (Sample Pack SS-2A, 500-ml volume, Terraoka) were filled with the soil, and the ceramic tube was placed in the center. A 10-ml syringe was inserted into the 200-µl tip. The entire containers were wrapped with aluminum foil. The artificial root exudate (ARE) consisted of primary metabolites such as sugars and amino acids (Schulz-Bohm et al., 2015). α-Tomatine (4 mM) dissolved in dimethyl sulfoxide (DMSO) and the mixed ARE solution were added into the ceramic tube. All solutions contained the same ratio of DMSO (1%). One milliliter of the solution was added daily for 14 days using a Pasteur pipette. Soil was sampled at 14 days using a cork poler (part number: 5-5388-04, cork poler saw blade, drilled diameter: 4.0 mm, Nonakarikaki Co., Ltd.). Soils were sampled at 5, 10, 15, 20, 25, and 30 mm from the center of the mullite ceramic tube.

2.5 | Extraction of metabolites from soils

The extraction of metabolites was performed as previously described (Nakayasu, Ohno, et al., 2021). Two milliliters of 25% (v/v) acetonitrile containing .1% (v/v) formic acid was added to the soil samples and vortexed for 1 min. Then, the samples were sonicated for 15 min, followed by centrifugation at 10,000 $\times g$ for 5 min. The supernatants were collected in a 2-ml tube, and the extraction was performed thrice. In total, 1.5 ml of extract was obtained from each soil sample. The extracts were dried and solidified in a decompression centrifuge (EYELA) at 80°C for 3 h, and the resulting solid was dissolved in 500 µl of methanol. Samples were filtered through a .20-mm Minisart RC4 filter (Sartorius, Göttingen, Germany) and analyzed using liquid chromatography–mass spectrometry (LC–MS). The concentrations of α-tomatine and tomatidine were calculated per unit weight of Toyoura sand. α-Tomatine and tomatidine were analyzed using Acquity ultra-high-performance LC (UPLC) H-Class/Xevo TQD system (Waters). Samples were injected into Acquity UPLC BEH C18 columns (1.7 µm, 2.1 × 50 mm; Waters) at 40°C. The mobile phases were water containing .1% (v/v) formic acid (solvent A) and acetonitrile (solvent B). The elution programs were set as 10–90% solvent B from 0 to 10 min (linear gradient), 90% solvent B from 10 to 12 min, 100% solvent B from 12 to 16 min, and 10% solvent B from 16 to 21 min. The flow rate was .2 ml min^{-1}. Mass spectra were acquired using the positive electrospray ionization mode with the following settings:

cone voltage, 30 V; capillary voltage, 3.15 kV; source temperature, 150°C; desolvation gas temperature, 400°C; and nebulizer and desolvation N_2 gas flow rates, 50 and 800 L h^{-1}, respectively. α-Tomatine and tomatidine were detected using selected ion recording modes at 1034.7 and 416.5 *m/z*, respectively. Data analysis was performed using MassLynx v.4.1 software (Waters). The contents of α-tomatine and tomatidine were estimated based on the peak areas compared with those in the calibration curves constructed using known concentrations of authentic compounds.

2.6 | DNA extraction, PCR, and sequence analysis

DNA was extracted from bulk soils and roots using DNeasy PowerSoil Kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions. DNA concentration was measured using Qubit dsDNA HS Assay Kit and Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted DNA was then diluted with MilliQ water to 2 ng/μl. The V4 region of the 16S rRNA genes was amplified using PCR with the following forward and reverse primers: 515F (5′-ACACTCTTTCCCTACACGACGCTCTTC-3′) and 806R (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-GGACTACHVGGGTWTCTAAT-3′). This amplification was performed using KOD FX Neo (TOYOBO, Osaka, Japan) in technical triplicate. The following PCR program was used for the soil samples: 94°C for 2 min, followed by 20 cycles of 98°C for 10 s, 50°C for 30 s, and 68°C for 30 s. For the root samples, mitochondrial- and chloroplast-specific peptide nucleic acids (mPNA [N-term-GGCAAGTGTTCTTCGGA-C-term] and pPNA [N-term-GGCTCAACCCTGGACAG-C-term], respectively [PANAGENE Inc, Daejeon, Republic of Korea]) were added to the PCR mixture. The PNA annealing step at 78°C for 10 s was performed before the annealing step at 50°C for 30 s in the PCR cycle to prevent the amplification of tomato mitochondrial and plastid sequences (Lundberg et al., 2013). For each sample, 5 μl each of the first PCR products from three replicates was collected and mixed to obtain a total PCR product of 15 μl. The PCR products were purified using AMPure XP (Beckman Coulter, Danvers, MA, USA), and residual primers, enzymes, and other contaminants were removed according to the protocol. Two repetitions of the second PCR were performed for each first PCR product. In the second PCR, the common sequences and index sequence were added to the first PCR product. In the second PCR, the following primers were used to attach the adapter (forward primer inserts the i5 index sequence and reverse primer inserts the i7 index sequence into the full length sequence) (Illumina, San Diego, CA, USA): forward primer (D50X; 5′-AATGATACGGCGACCACCGAGATCTACAC [i5 index] ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′) and reverse primer (D7XX; 5′-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC [i7 index] ATCTCGTATGCCGTCTTCTGCTTG-3′). The following PCR program was employed for all samples: 94°C for 2 min, followed by 9–10 cycles of 98°C for 10 s, 59°C for 30 s, and 68°C for 30 s. Overall, 12.5 μl of each second PCR product was mixed to obtain a total PCR product of 25 μl. The PCR products were purified using Agencourt AMPure XP in the same manner as the first PCR products, and the concentration was measured using Qubit. The prepared 16S amplicon sequence was acquired as 2 × 250-bp paired-end sequences using Miseq (Illumina) and was analyzed by FASMAC Co., Ltd. The dataset is publicly available at the DNA Data Bank of Japan (DDBJ) (https://www.ddbj.nig.ac.jp) (DRA015675 and DRA015694).

Amplicon sequence data analysis was performed using QIIME2 platform version 2021.11 (Bolyen et al., 2019). For quality filtering of all pair-end reads, the q2-dada2 plugin in QIIME2 was used to trim the first 20 bases and those after the 200th base. DADA2 algorithm was used to construct error-corrected amplicon sequence variants (ASVs) from the trimmed reads (Callahan et al., 2016). MAFFT was used to align the obtained ASVs, and FastTree2 was used to construct the phylogenetic trees (Katoh et al., 2002; Price et al., 2010). After filtering the chloroplastic and mitochondrial sequences, the α- and β-diversity were estimated based on the subsampled ASV dataset with a depth of 31,000 for each pot-cultivated tomato sample, a depth of 11,000 for the model system sample, and a depth of 11,000 for the combined pot-cultivated tomato and model system samples. Each ASV was assigned to a taxonomic group using the NaïveBayes classifier from SILVA (release 138.1, Bokulich et al., 2018; Quast et al., 2013). Principal coordinate analysis (PCoA) was performed using q2-diversity plugin.

2.7 | Bacterial culture and *chemotaxis assay*

Sphingobium sp. RC1 isolated from tomato roots (Nakayasu et al., 2023) was used for chemotaxis assay. Bacterial strain was cultured in lipid tryptone yeast extract glucose medium (Bai et al., 2015) at 28°C 180 rpm for 2 days to reach the exponential growth phase. The chemotaxis assay was performed as previously described with modifications (Mazumder et al., 1999). A disposable 200-μl pipette tip (Watoson) was used as the chamber for holding 100 μl of bacterial solution with $OD_{600} = .1$. A disposable 127-mm Calibrated Pipet of a 10-μl capacity and a .50 internal diameter (Funakoshi) was attached to a 1-ml syringe (Terumo) by placing a 2-μl tip in between. α-Tomatine (100 μM) or sterilized water (including methanol) was applied to the capillary by using the attached syringe. The capillary tip was inserted into bacterial cell suspension and incubated for 2.5 h. Excess bacterial suspension was removed, and cell suspension was diluted to 10^{-3}. The diluted suspension was placed onto tryptone yeast extract glucose medium containing 1.5% agar (Bai et al., 2015), and the number of colony formation units (CFUs) was counted after incubating for 3 days. Data are also displayed as relative attraction rate, which are the quotient of the cells in the test capillary divided by those in the control capillary.

2.8 | Collection and analysis of public data on tomato rhizosphere microbiome

A literature search was conducted via Google Scholar using the keywords "tomato," "rhizosphere," and "16S." Data from four previously published studies (BioProject accession numbers: PRJEB40313,

PRJNA507021, PRJNA754706, and PRJNA485233) that met the following criteria were used for meta-analysis (Table S3): (1) bulk soil samples corresponding to rhizosphere samples were available, (2) sequencing instrument and paired-end data were the same as those used in our study (Illumina Miseq), (3) 16S rRNA target regions varied across studies, and (4) study locations varied geographically across studies. Raw sequence data (FASTQ files) were downloaded from Sequence Read Archive (SRA) based on the accession number, and sample metadata were obtained from SRA Run Selector.

Bioinformatics processing of the raw sequences was performed using QIIME2 version 2021.2 (Bolyen et al., 2019). Primer sequences from each study were removed from the sequence data using cutadapt (Martin, 2011) and were then denoised using DADA2 (Callahan et al., 2016) to generate ASVs. The raw sequence data of PRJNA754706 were a prejoined sequence; therefore, these sequences were denoised using Deblur (Amir et al., 2017). Taxonomy was assigned to the sequences using the q2-feature-classifier plugin (Bokulich et al., 2018) Naive Bayes classifier, which was pretrained using 99% operational taxanomic unit sequences from each primer target region of the reference sequence based on the SILVA rRNA database release 138 (Quast et al., 2013). After filtering out mitochondrial and chloroplast sequences, samples with total read counts of <5000 were excluded. For differential abundance analysis of bacterial taxa at the genus level between bulk soil and rhizosphere, we used ALDEx2 (Fernandes et al., 2013, 2014) with a false discovery rate cutoff of .05.

2.9 | Statistical analysis

Statistical analysis was performed using R (v. 4.0.3). Tukey's HSD test was performed using the R package multcomp (Hothorn et al., 2008). A comparison analysis of microbiota between the roots of tomato plants cultivated inside pots and bulk soil was performed using linear discriminant analysis effect size (LEfSe) to identify differentially abundant taxa at the genus level (Segata et al., 2011). For two-way analysis of variance (ANOVA) and pairwise Wilcoxon tests, *P* values of <.05 indicated statistical difference using default parameters, and the LDA effect size was >2. Permutational multivariate analysis of variance (PERMANOVA) was performed using the q2-diversity plugin. *T* test was carried out using t.test function of R. Hierarchical cluster and heatmap analyses of the microbial community structure were performed using R with the "minkowski" distance measure and "average" clustering algorithm (Kolde, 2012). The map of Japan was constructed using the R package NipponMap (https://CRAN.R-project.org/package=NipponMap).

3 | RESULTS

3.1 | Cultivation of tomato in pots with three different soils

In our previous study using the *jre4* mutant, which secrete lower amount of α-tomatine, it was revealed that α-tomatine secreted from tomato roots enriched *Sphingobium* (Nakayasu, Ohno, et al., 2021); however, this phenomenon was observed in a particular microbial community obtained from a field. In the current study, to investigate the commonality of tomato root-associated bacterial communities, tomatoes were cultivated in Agripots filled with Toyoura sand and three different soils collected from different locations in Japan as microbial inoculants (Figure S1a). The inorganic nutrients in each field soil were different (Table S1); however, a culture medium was added

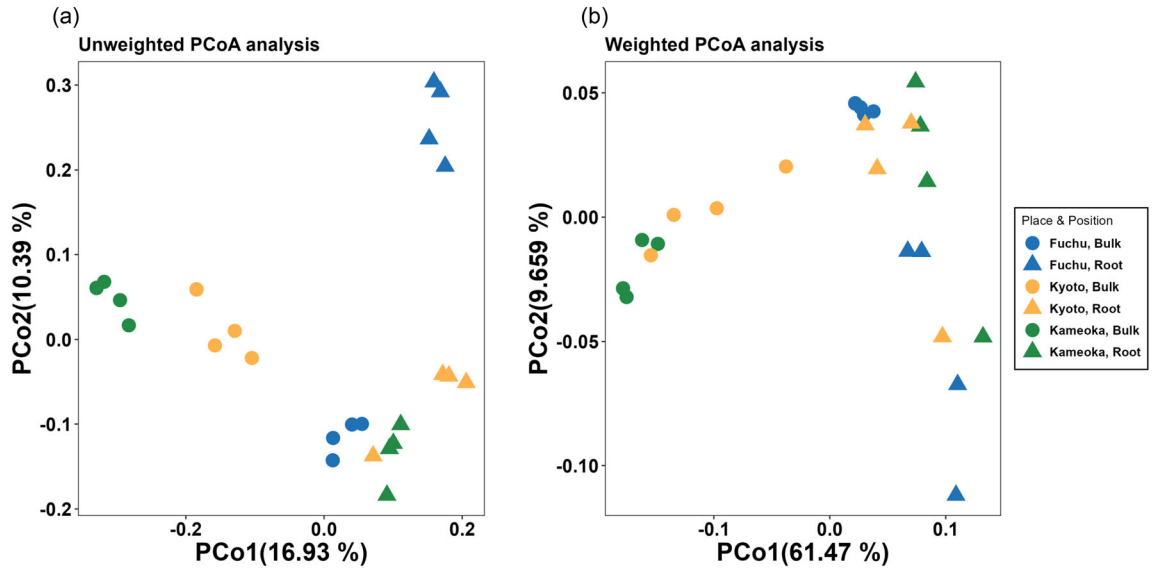

FIGURE 1 β-Diversity of bacterial communities in bulk soil and tomato roots in three different field soils using principal coordinate analysis (PCoA). PCoA plots of bulk soils and roots obtained from pot cultivation experiments based on the UniFrac distance. (a) Unweighted UniFrac-based PCoA. (b) Weighted UniFrac-based PCoA.

to identical volumes to ensure that bacterial communities were not affected by nutrient deficiencies. There were no differences in the above-ground weights, but the root weights showed differences among the three inoculants, with a declining trend observed in Kyoto soil and a significant increase observed in Kameoka soil (Figure S1b,c).

The bacterial community was analyzed in both bulk soil and tomato roots after cultivation. Evaluation of α-diversity using Faith's phylogenetic diversity (Faith's PD) index showed that the microbial diversity was distinct among the three soil types and sampling parts, but Tukey post hoc analysis indicated that a significant difference between bulk and root-associated bacterial communities was observed only in Kyoto soil (two-way ANOVA, $P < .05$; Tukey post hoc analysis, $P < .05$; Figure S2; Table S4). Based on PCoA, the bacterial community structure was significantly different among the three bulk soil samples (PERMANOVA, $P < .05$; Figure 1 and Table S5). Both weighted and unweighted UniFrac distance metrices revealed that the root-associated bacterial communities were significantly different from those of bulk soil (PERMANOVA, $P < .05$; Figure 1 and Table S5), and the unweighted UniFrac distance showed that the root-associated bacterial communities were distinctive depending on the inoculated soils. LefSe analysis was performed to identify the bacterial genera that were significantly more abundant in root-associated bacterial communities than in bulk soil and showed that four bacterial genera, *Sphingobium*, *Bradyrhizobium*, *Cupriavidus*, and *Rhizobacter*,

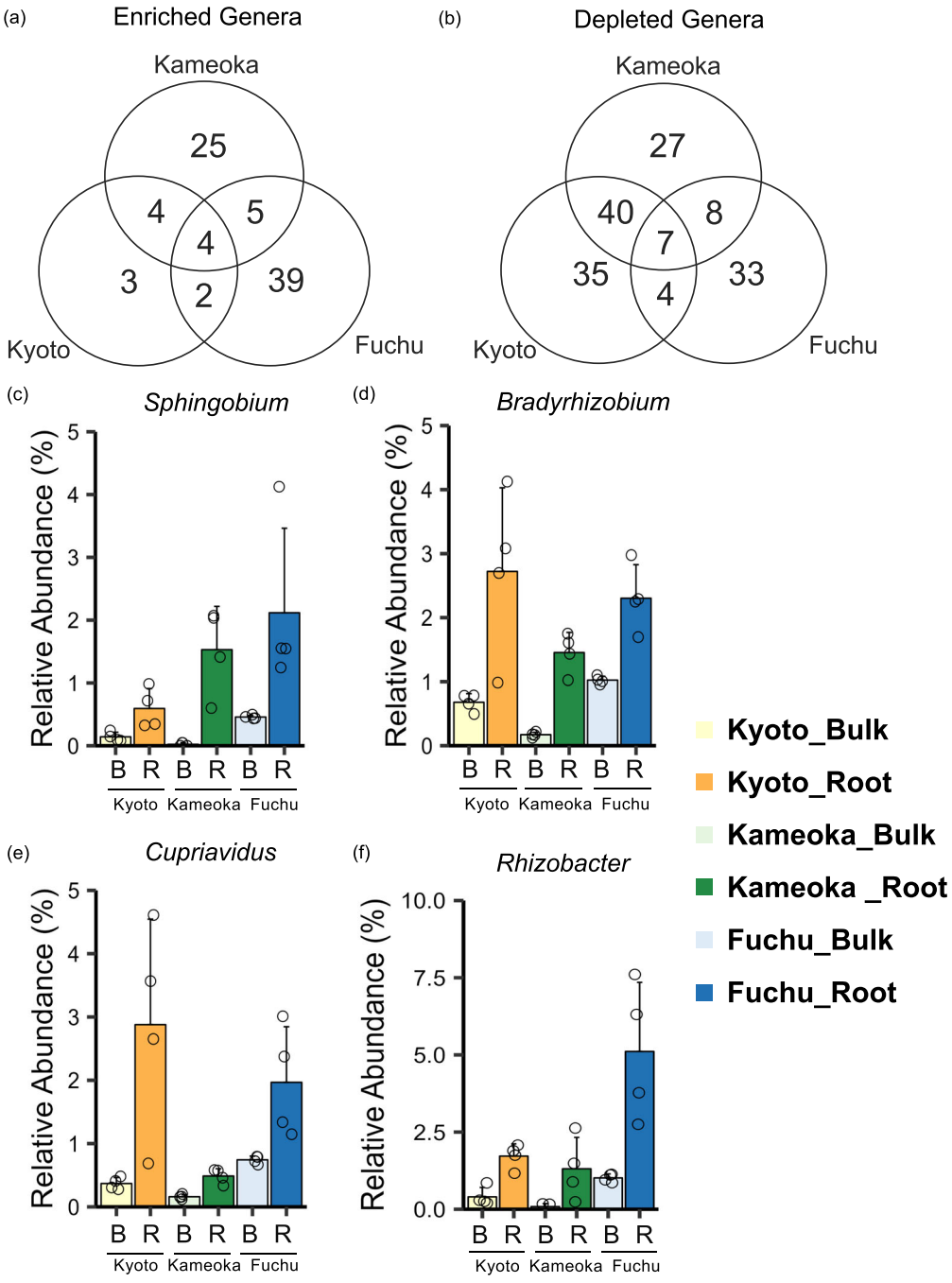

FIGURE 2 Comparison of the bacterial communities of bulk soil and tomato roots. Venn diagrams illustrate the overlap of enriched (a) or depleted (b) bacterial genera in Kameoka, Kyoto, and Fuchu field soils. The numbers of bacterial genera are shown in the circles. (c–f) The relative abundance of four genera commonly enriched in tomato roots. (c) The relative abundance of *Sphingobium*. (d) The relative abundance of *Bradyrhizobium*. (e) The relative abundance of *Cupriavidus*. (f) The relative abundance of *Rhizobacter*. Statistics analysis was performed using LEfSe (adjusted $P < .05$). Error bars indicate standard deviations (all samples, $n = 4$).

were commonly enriched in the root-associated bacterial communities when grown in soil with three different communities (Figure 2a and Table S6). In addition, seven bacterial genera were depleted in the root-associated bacterial communities (Figure 2b and Table S6).

3.2 | Rhizosphere analysis using an artificial root system

To analyze the enrichment of root-associated bacteria in the vicinity of tomato roots, we constructed a pseudo-rhizosphere system using a mullite ceramic tube as an artificial root (Figure S3). We used Kameoka field soil as the bacterial resource because it has been employed as a model in our previous studies (Nakayasu, Ohno, et al., 2021; Okutani et al., 2020; Shimasaki et al., 2021). ARE was added to the artificial roots to mimic root exudation from tomato roots. When ARE with α-tomatine was added, α-tomatine concentration gradient was formed with maximum concentration at a distance of 5 mm from the artificial root, which was in the closest compartment to the artificial root. Soil microorganisms degrade α-tomatine into tomatidine, which also enriches the relative abundance of Sphingomonadaceae in vitro (Nakayasu, Ohno, et al., 2021). Consequently, tomatidine was detected in soil with a concentration gradient similar to that of α-tomatine. Both α-tomatine and tomatidine concentrations showed a decreasing trend toward the outside of the container (Figure 3a). α-Tomatine and tomatidine were detected until a distance of 15 and 20 mm from the artificial root, respectively. The concentration of α-tomatine was within the same range as that of the tomato rhizosphere in the field (ranging from 10–300 nmol g soil^{-1}, depending on the growth conditions) (Nakayasu, Ohno, et al., 2021). The number of soil microorganisms was assessed by enumerating CFUs. An increase in CFU counts was observed in soil samples near the artificial root. The number of CFUs was significantly higher in soil with added ARE containing α-tomatine than in that with added ARE containing DMSO as a control (Tukey's HSD test, $P < .05$; Figure 3b). These results suggested that ARE and α-tomatine exudation from the mullite ceramic tube formed a condition that mimicked the tomato rhizosphere.

3.3 | Bacterial community analysis using pseudo-rhizosphere device

PCoA analysis based on the weighted UniFrac distance showed that the microbial communities were significantly different depending on the distance from the artificial root (PERMANOVA, $P < .05$; Figure 4a and Table S7). PCoA analysis based on unweighted UniFrac distance revealed that the bacterial communities of α-tomatine-treated soil at distances of up to 10 mm from the artificial root were significantly different from those of the control (PERMANOVA, $P < .05$; Figure 4b and Table S7). In particular, a distinction in community composition was observed until a distance of 15 mm, where α-tomatine was detected, but bacterial communities in α-tomatine-treated soil and the control were comparable at a distance of 20 mm or more from the artificial root.

We analyzed four bacterial genera that are commonly enriched in tomato root-associated bacterial communities. The relative abundance of *Sphingobium* in α-tomatine-treated soil was approximately 6-fold higher than that in the control soil at a distance of 5 mm (Figure 5). The relative abundance of *Sphingobium* gradually decreased depending on the distance from the artificial root. The relative abundances of *Bradyrhizobium*, *Cupriavidus*, and *Rhizobacter* did not increase in the vicinity of the artificial root in α-tomatine-treated soil (Figure 5). We then tested whether *Sphingobium* is attracted to α-tomatine. Chemotaxis assays using capillaries demonstrated that *Sphingobium* sp. isolated from tomato roots showed a positive chemotaxis to α-tomatine (Figure 6).

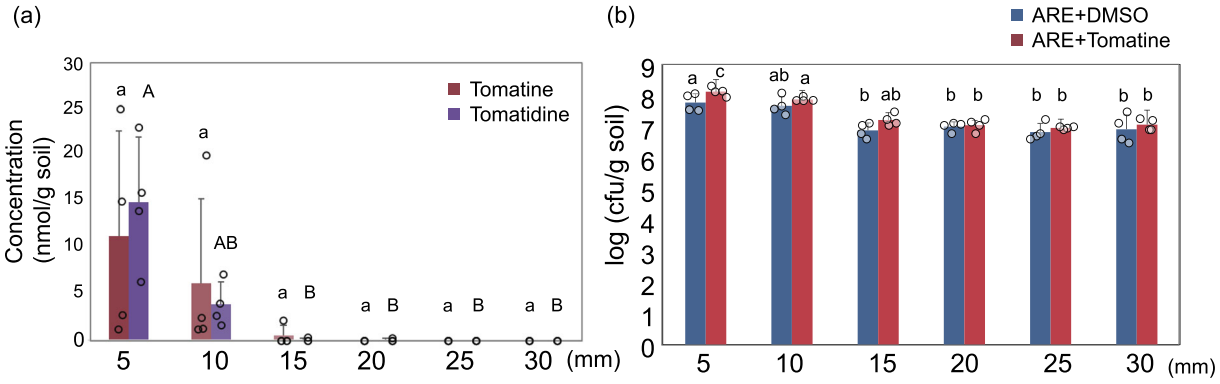

FIGURE 3 Contents of α-tomatine and tomatidine as well as colony formation unit (CFU) count in the soil depends on the distance of the pseudo-rhizosphere system. α-Tomatine (4 mM) was used for this experiment. (a) The concentration of α-tomatine and tomatidine in soil at a distance of every 5 mm from an artificial root. Red bars represent the contents of α-tomatine; purple bars represent the contents of tomatidine. Different letters (a for tomatine; A–B for tomatidine) indicate statistically significant differences ($P < .05$) for the contents of tomatine and tomatidine, respectively, using Tukey's test. (b) CFU count in the soil at a distance of every 5 mm from an artificial root. Blue bars represent conditions of ARE exudation, and red bars represent conditions of ARE with α-tomatine exudation. Different letters (a–c) indicate statistically significant differences ($P < .05$) for the number of colony formation units, using Tukey's test. Error bars indicate standard deviations (all samples, $n = 4$).

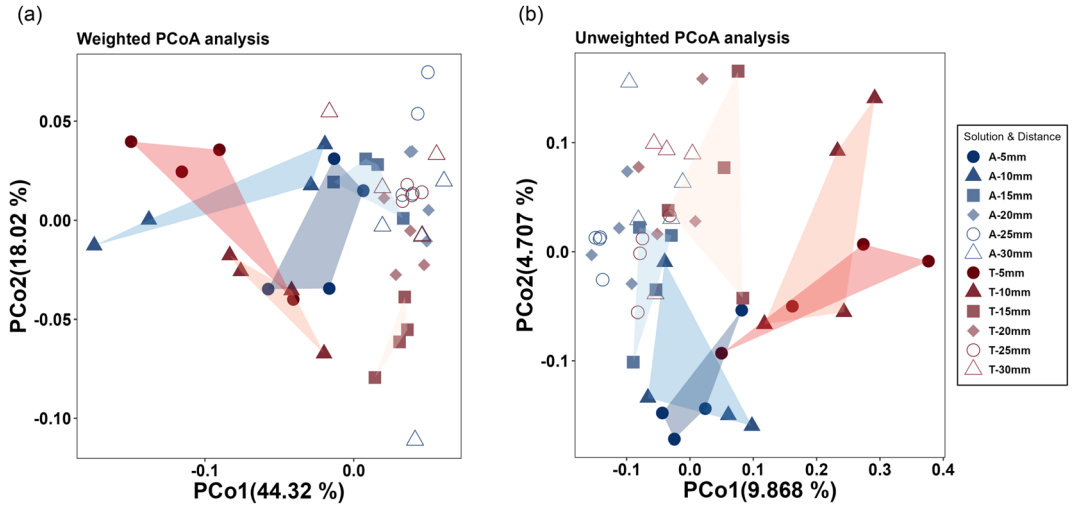

FIGURE 4 β-Diversity of bacterial communities in the soil of the pseudo-rhizosphere system using principal coordinate analysis (PCoA). PCoA plots of UniFrac distance obtained from the bacterial communities of sampling spots at distances ranging from 5 to 30 mm from an artificial root. (a) Weighted UniFrac-based PCoA. (b) Unweighted UniFrac-based PCoA. Only ARE-exudated condition is indicated in blue. ARE-containing α-tomatine exudated condition is indicated in red. The polygon is shown from 5 to 15 mm from an artificial root.

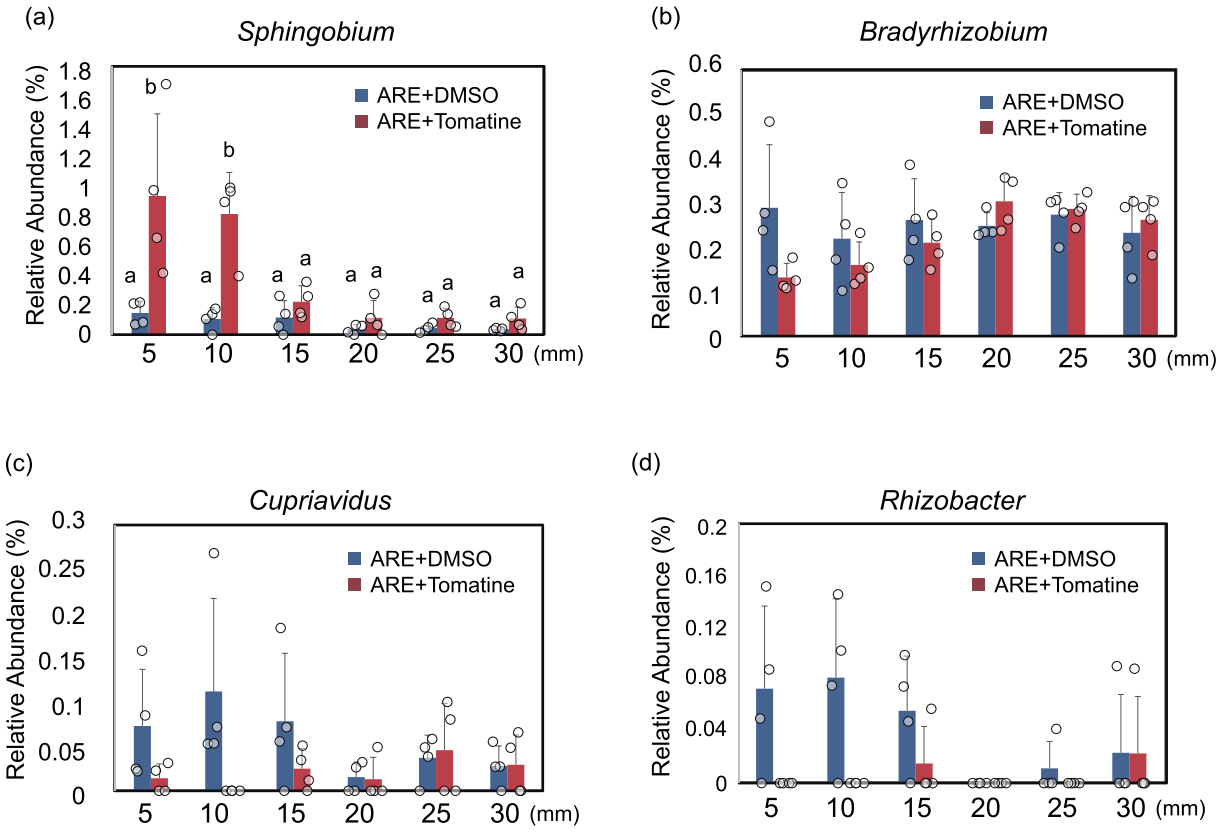

FIGURE 5 Relative abundance of four bacteria in the pseudo-rhizosphere system. The relative abundance of four bacterial genera (*Sphingobium*, *Bradyrhizobium*, *Cupriavidus*, and *Rhizobacter*) that were significantly increased in tomato roots regardless of field soil type was examined. Only ARE-exudated condition is indicated in blue. ARE-containing α-tomatine exudated condition is indicated in red. Different letters (a,b) indicate statistically significant differences ($P < .05$) using Tukey's test. Error bars indicate standard deviation (all samples, $n = 4$).

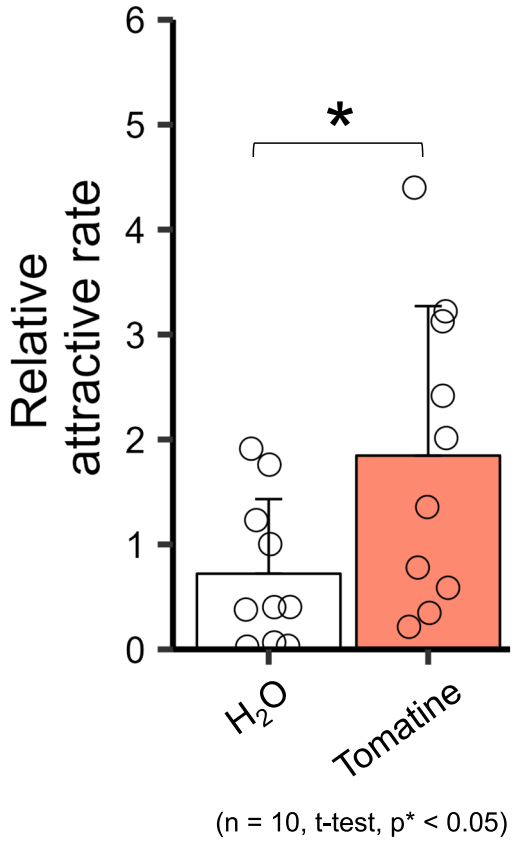

FIGURE 6 A comparison of the chemotactic response of *Sphingobium* sp. to α-tomatine. *Sphingobium* sp. RC1 showed positive chemotaxis to α-tomatine. Chemotaxis assayed with or without 100-μM chemo-attractants. Error bars indicate standard deviations (all samples, $n = 10$). Statistics analysis was performed using *t* test (adjusted $P < .05$).

3.4 | Comparison of microbiota between pot-cultivated tomato plants and pseudo-rhizosphere system

We compared the tomato root-associated bacterial communities with the bacterial communities formed in the pseudo-rhizosphere system. Hierarchical cluster analysis of relative abundance in bacterial communities showed a difference between the two experimental conditions (Figures 7, S4, S5, and S6; Table S8). The results of PCoA using weighted and unweighted UniFrac distances showed that the conditions between pot cultivation and the pseudo-rhizosphere system were evidently different (Figure 8a,b, S7; Table S9). Although differences were observed between the experimental conditions, the transition of bacterial communities from 30 to 5 mm in the artificial root system and that from bulk soil to root in pot cultivation were indicated by the second axis of PCoA (Figures 8a and S7a). Unweighted PCoA analysis revealed that changes in bacterial communities in α-tomatine-treated soil showed a similar trend to that of changes in tomato root-associated bacterial communities, as represented by the second axis of PCoA (Figures 8b and S7b). The tomato root-associated bacterial community of Kameoka field soil was closer to the 5-mm than to the 30-mm communities in ARE-added soil, whereas the bulk soil bacterial community was closer to the 30-mm than to the 5-mm communities in α-tomatine-treated soil (Figure 8c,d). Altogether, these results suggest that the experimental conditions predominantly affect the bacterial communities.

3.5 | Meta-analysis of the tomato rhizosphere microbiome

To further support the association between tomato and *Sphingobium*, we performed meta-analysis of tomato rhizosphere microbial communities using the publicly available microbiome data. In four independent studies (Table S3) from different countries using cultivated and wild tomato (*S. pimpinellifolium*), we identified bacterial genera that were significantly enriched in the rhizosphere. These data showed that *Sphingobium* was the only genus that was commonly enriched in all four datasets (Figures 9 and S8).

We also analyzed three other bacterial genera that were commonly enriched in the tomato rhizosphere in the pot cultivation experiment. The abundance of *Bradyrhizobium*, *Cupriavidus*, and *Rhizobacter* in the tomato rhizosphere varied depending on the experiment (Figure S8). These results suggest that the α-tomatine-mediated tomato–*Sphingobium* interaction is conserved in the tomato rhizosphere irrespective of bulk soil community.

4 | DISCUSSION

PSMs secreted from plant roots shape root-associated microbial communities (Jacoby et al., 2021; Massalha et al., 2017); however, most studies have evaluated PSM–microbiota interactions in a particular soil and whether a difference in the initial soil microbial communities affects these interactions needs further evaluation. We previously reported that tomato roots secrete α-tomatine, which modulates the tomato rhizosphere bacterial community, particularly by enriching *Sphingobium*, and that *Sphingobium* isolated from α-tomatine-treated soil utilizes α-tomatine as a carbon source (Nakayasu, Ohno, et al., 2021). Because we used only one tomato field in Kameoka, Japan, the following remain unclear: (1) whether the enrichment of *Sphingobium* is commonly observed in other fields and (2) whether bacterial genera other than *Sphingobium* are enriched in tomato root-associated bacterial communities. To answer these questions, we cultivated tomato plants using soil consisting of three different microbial inoculants from field soils. Both bulk soil and root-associated bacterial communities showed different trends in microbiota depending on the added soil microbial inoculants, which is in line with previous studies reporting that the microbial communities of bulk soil predominantly affect the root-associated microbiota (Thiergart et al., 2020; Tkacz et al., 2015). Four bacterial genera, *Sphingobium*, *Bradyrhizobium*,

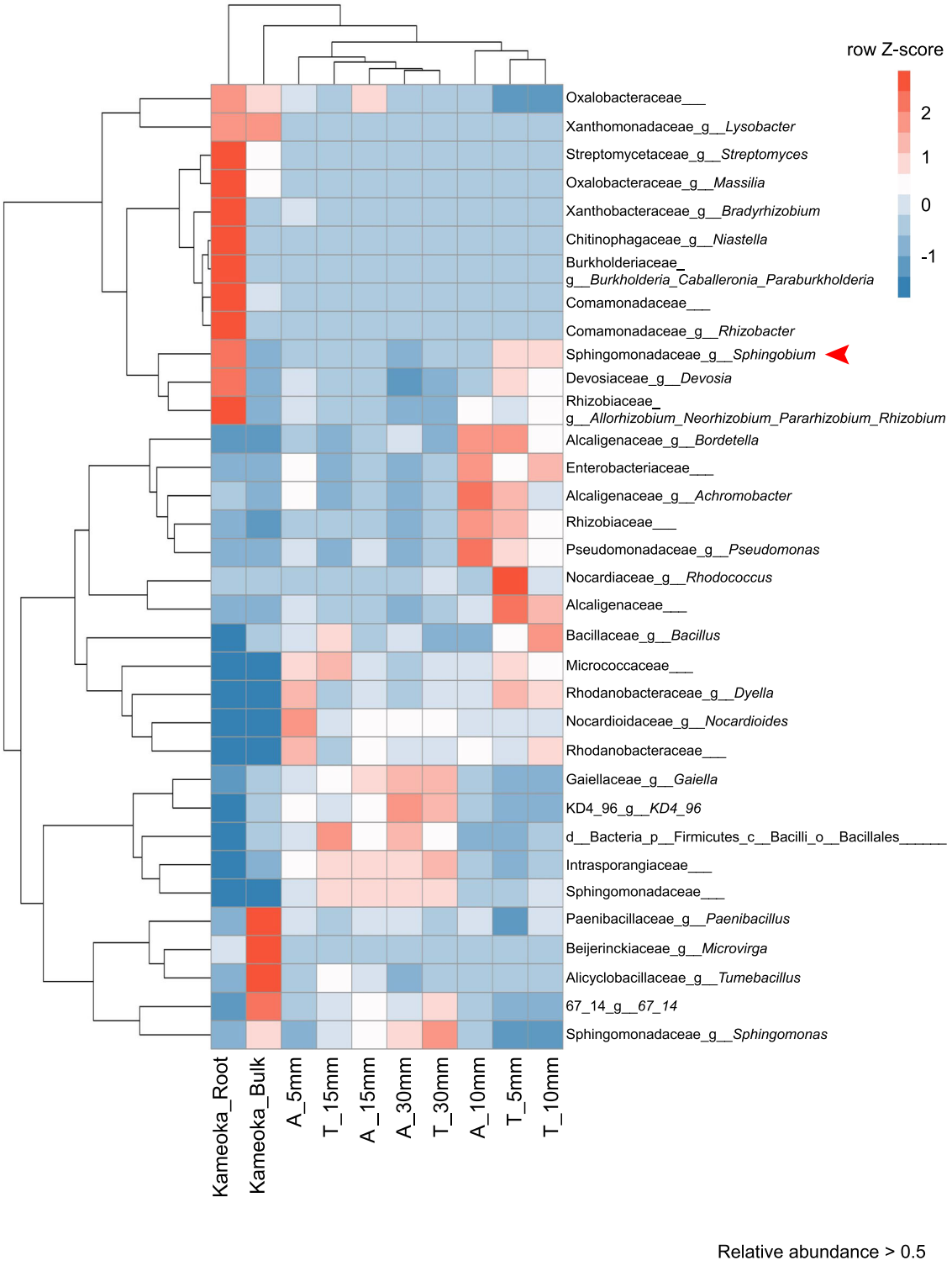

FIGURE 7 Hierarchical clustering and taxonomic annotation of bacterial 16S rRNA marker gene sequenced species. Comparison of the bacterial communities in bulk soil and tomato roots in the experimental pots and soil of the pseudo-rhizosphere system. Heatmaps of the relative abundances of bacterial genera (the mean sample relative abundance was >.5). Hierarchical clustering with heatmap construction was performed using the R package pheatmap.

Cupriavidus, and *Rhizobacter*, were commonly enriched in tomato root-associated bacterial communities in the pot experiments. *Sphingobium* was enriched in the rhizosphere of tomato plants in all meta-analyses, whereas the relative abundance of the other three genera varied, suggesting a tight interaction between tomato and *Sphingobium*.

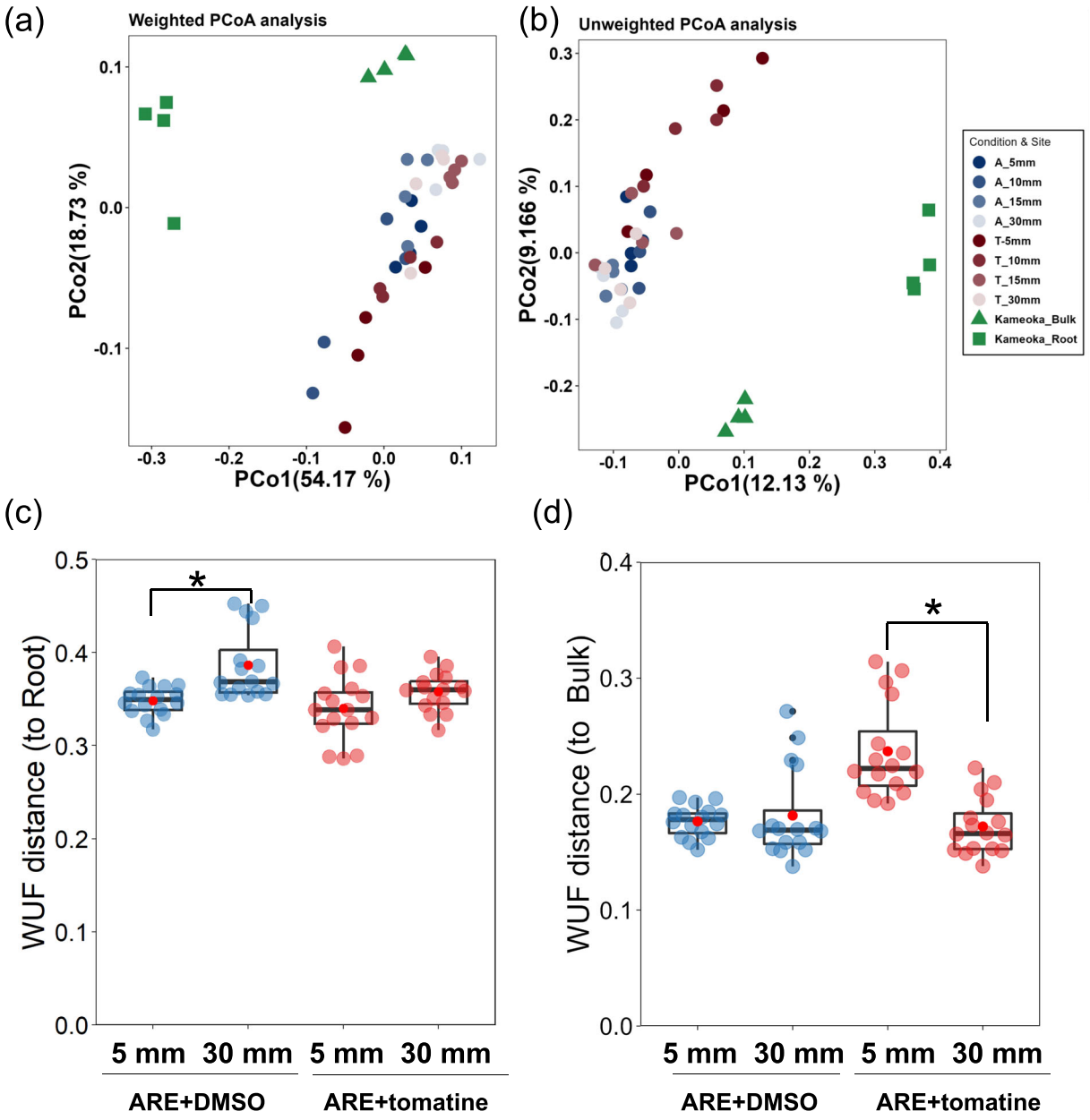

FIGURE 8 β-Diversity of bacterial communities in bulk soil and root samples of pot-grown tomatoes under field soil treatment conditions as well as soil samples of the pseudo-rhizosphere system using principal coordinate analysis (PCoA) (a) weighted UniFrac-based PCoA. (b) Unweighted UniFrac-based PCoA. Comparison of the bacterial communities in tomato roots with those cultivated in the experimental pots and soil of the pseudo-rhizosphere system. Weighted UniFrac distance as indices of dissimilarity between the soil bacterial community in the pseudo-rhizosphere system and the tomato root endophytic community grown in pots with the addition of soil from the Kameoka field. Box plots showing mean and variance of average pairwise weighted UniFrac distances between (c) root and pseudo-rhizosphere and between (d) bulk soil and pseudo-rhizosphere. Asterisks indicate statistically significant differences ($P < .05$, Wilcoxon rank-sum test). Error bars indicate standard deviation (all samples, $n = 4$). Red circles indicate the mean.

Artificial root methods using porous materials enable researchers to investigate the effect of specific compounds in root exudates in the rhizosphere (Buckley et al., 2022; Zhang et al., 2019) and can be applied to establish a gradient of metabolites that mimic rhizosphere environmental conditions. To address whether α-tomatine shapes the bacterial communities in the tomato rhizosphere, we constructed a pseudo-rhizosphere system using mullite ceramic tubes as artificial roots. A concentration gradient of α-tomatine similar to that in the tomato rhizosphere and higher CFU counts in the proximity of the roots were observed in our system, indicating that this pseudo-rhizosphere device can be used to investigate PSM-mediated plant–microbe interactions in the rhizosphere. The relative abundance of *Sphingobium* was the highest at a distance of 5 mm and gradually decreased with decreasing concentrations of α-tomatine, but the

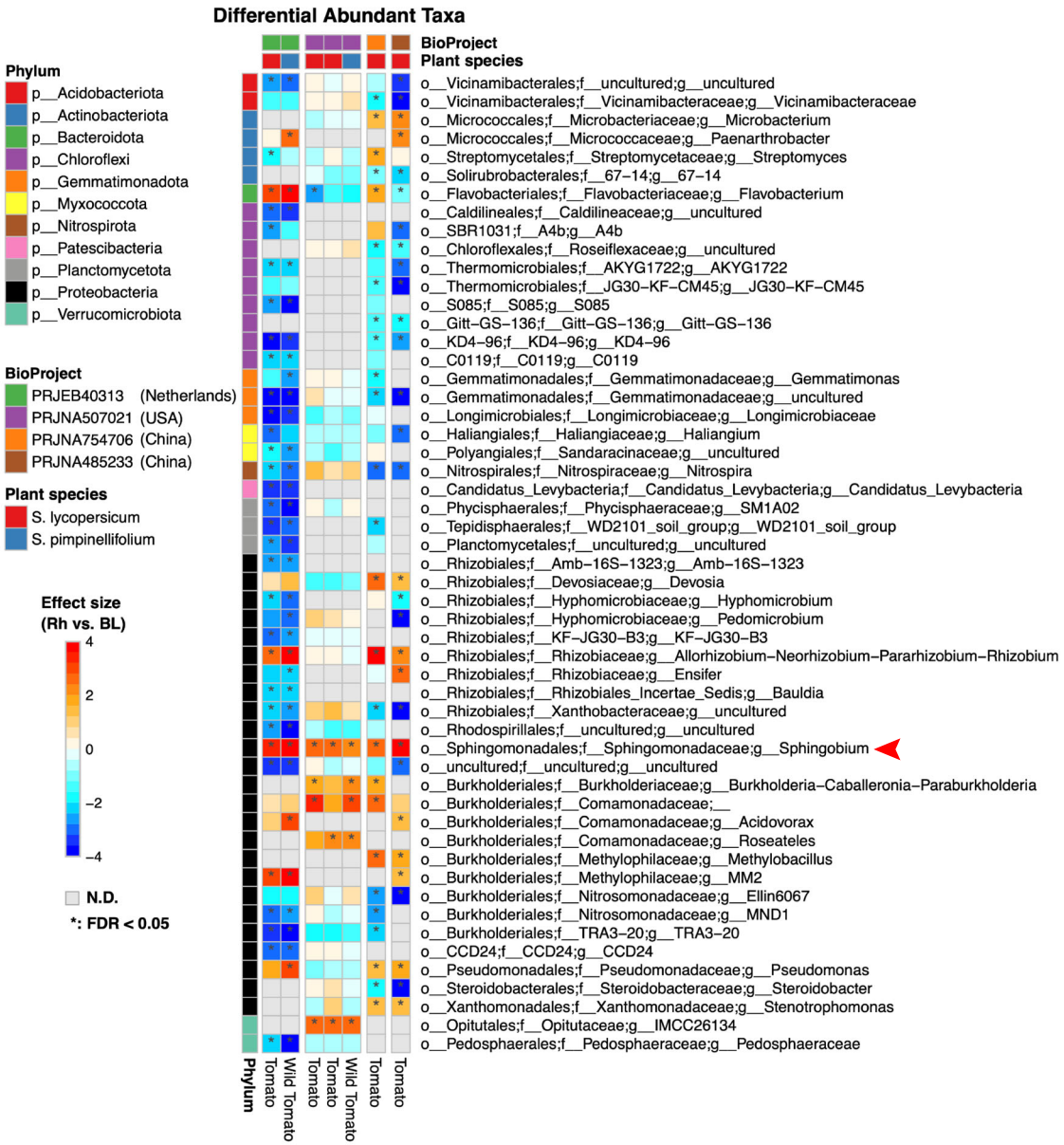

FIGURE 9 Differential abundant bacterial taxa between bulk soil and tomato rhizosphere in publicly available microbiome data. Representative samples of untreated and healthy plots from each study were used for statistical analysis. Differential abundant bacterial genera between the bulk soil and rhizosphere were identified using ALDEx2. The heatmap shows the ALDEx2-estimated effect sizes for bacterial taxa that were significantly different in two or more comparison groups (FDR < .05). Red and blue indicate enriched and depleted taxa, respectively. Gray indicates that the bacterial taxon was not detected in that study. An arrowhead indicates *Sphingobium*. The average relative abundance for each experiment was used. Rh, rhizosphere; BL, bulk soil.

relative abundance of *Bradyrhizobium*, *Cupriavidus*, and *Rhizobacter* did not increase in soil samples near the artificial root-secreting ARE with increasing concentrations of α-tomatine. In fact, *Sphingobium* sp. isolated from tomato roots exhibited a positive chemotaxis to α-tomatine. A recent study has shown that *Sphingobium* sp. isolated from Beachgrass has a root elongation effect (Boss et al., 2022). α-Tomatine enriches *Sphingobium*, and it may benefit plants. In contrast, the relative abundances of *Cupriavidus* and *Rhizobacter* showed an increasing trend around the artificial root that secreted only ARE, which included primary metabolites, such as sugars, amino acids, and organic acids. This suggests that the enrichment of *Cupriavidus* and *Rhizobacter* was at least partly stimulated by the compounds in ARE, whereas α-tomatine may suppress the chemotactic activity. *Cupriavidus* and *Rhizobacter* have been observed in the rhizospheres of a wide range of plants other than tomatoes; for instance, *Cupriavidus* symbiosis with mimosa and its colonization of the roots of maize, sugarcane, agave, and sorghum have been reported (Estrada-de los Santos et al., 2011; Liu et al., 2011). Moreover, *Rhizobacter* is enriched in the sugarcane rhizosphere under biochar and nitrogen fertilization-treated conditions (Pang et al., 2022), suggesting the roles of primary

metabolites commonly secreted from roots in the enrichment of these bacterial genera. The relative abundance of *Bradyrhizobium* did not show any change with the addition of ARE, but it decreased in the soil close to the artificial root-secreting α-tomatine. These results suggest that root-secreted metabolites not used in ARE or other properties of root system enriched *Bradyrhizobium* in our analysis. *Bradyrhizobium* is known to be enriched in soybean roots, and treatment of the seed exudates containing high amounts of sugars and amino acids leads to *Bradyrhizobium* proliferation (Iizuka et al., 2002). In particular, *Bradyrhizobium* is strongly attracted to dicarboxylic acids and amino acids, such as glutamic acid and aspartic acid (Barbour et al., 1991). The ARE used in this study consisted of 14 compounds, which do not fully represent the diversity of metabolites in the root exudates, although they are found abundantly in root exudates. This device can be used to evaluate the effects of a more complex mixture of metabolites in the modulation of rhizosphere microbiota by adding root or seed exudates containing higher concentrations of sugars and amino acids. In addition to PSMs, root architecture plays a crucial role in shaping the microbiome. Therefore, it is necessary to develop a pseudo-rhizosphere system that incorporate different root properties to better understand the influence of roots on recruiting and shaping rhizosphere microbiome in future studies.

In conclusion, this study showed that four bacterial genera, namely, *Sphingobium*, *Bradyrhizobium*, *Cupriavidus*, and *Rhizobacter*, were commonly enriched in tomato root-associated bacterial communities when three different Japanese field soils were used as microbial inoculants. Among these genera, *Sphingobium* was the only genus enriched in the vicinity of the artificial root that secreted α-tomatine in a concentration-dependent manner. This pseudo-rhizosphere system, comprising a mullite ceramic tube secreting α-tomatine, along with the enrichment of *Sphingobium* in the tomato rhizosphere in public data with multiple species and bulk soil communities revealed a tight association between α-tomatine and *Sphingobium*. This pseudo-rhizosphere could be a valuable tool to evaluate the spatiotemporal distribution and functions of PSMs in plant–microbiota interactions in the rhizosphere.

AUTHOR CONTRIBUTIONS

Kyoko Takamatsu and Akifumi Sugiyama conceived and designed the research. Masaru Kobayashi, Kentaro Ifuku, Kazufumi Yazaki and Akifumi Sugiyama supervised the experiments. Kyoko Takamatsu and Masaru Nakayasu analyzed the tomato growth, tomatine content, and bacterial communities. Kyoko Takamatsu, Miwako Toyofuku and Fuki Okutani performed the pseudo-rhizosphere experiments. Shinichi Yamazaki and Yuichi Aoki performed the meta-analysis. Kyoko Takamatsu, Shinichi Yamazaki, and Akifumi Sugiyama wrote the article with contributions from all authors. Akifumi Sugiyama agreed to serve as the author responsible for contact and ensure communication.

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CONFLICT OF INTEREST STATEMENT

The Authors did not report any conflict of interest.

PEER REVIEW

The peer review history for this article is available in the Supporting Information for this article.

DATA AVAILABILITY STATEMENT

The 16S rRNA amplicon sequencing datasets supporting the results of this study are publicly available at the DNA Data Bank of Japan (https://www.ddbj.nig.ac.jp) (DRA015675 and DRA015694).

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SUPPORTING INFORMATION

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