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Biodegradation of isoprene by soil Actinomycetota from coffee-tea integrated plantations in a tropical evergreen forest

Toungporn Uttarotai [a,b], Terry J. McGenity [c], Sawannee Sutheeworapong [d], Wuttichai Mhuantong [e], Nuttapon Khongdee [a], Sakunnee Bovonsombut [f], Thararat Chitov [b,f,*]

[a] Department of Highland Agriculture and Natural Resources, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand
[b] Environmental Science Research Center, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[c] School of Life Sciences, University of Essex, Colchester CO4 3SQ, UK
[d] Systems Biology and Bioinformatics Laboratory, Pilot Plant Development and Training Institute, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand
[e] Enzyme Technology Research Team, Biorefinery and Bioproduct Technology Research Group, National Center for Genetic Engineering and Biotechnology, Pathumthani 12120, Thailand
[f] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

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ABSTRACT

Isoprene, a biogenic volatile compound emitted largely by plants, can form greenhouse gases when it reacts with atmospheric radicals. A significant amount of isoprene is absorbed into soil and can be degraded by soil microorganisms, but our understanding of the microbial biodegradation of isoprene in tropical ecosystems remains limited. This study investigated isoprene degradation by soil microbes indigenous to a tropical evergreen forest, focusing on those associated with coffee and tea plants grown as integrated crops and their genome characteristics in relation to their biodegradation capabilities. Following a 96-hour incubation with 7.2×10^5 parts per billion by volume (ppbv) of isoprene, soil samples exhibited degradation levels ranging from 11.95 % to 36.54 %. From these soils, bacterial isolates belonging to the genera *Rhodococcus* and *Gordonia* (Actinomycetota) were recovered. These isolates demonstrated high isoprene biodegradation activity (50.3 %–69.1 % over seven days) and carried the *isoA* gene associated with isoprene metabolism. According to genome analysis, the organization of genes in the *iso* cluster was homologous, and the encoded amino acid sequences were highly similar to those of previously known isoprene-degrading members of the same genera. These findings emphasized the contribution of these widespread isoprene-degrading bacterial genera in the biodegradation of isoprene and the role of their isoprene monooxygenases in modulating atmospheric isoprene flux.

1. Introduction

Isoprene, a biogenic volatile organic compound (BVOC), stands as the second most abundant atmospheric hydrocarbon after methane (Müller et al., 2008). It is produced by a variety of life forms, including plants, animals, bacteria, protists, and humans, resulting in an estimated 500 million tonnes of annual emissions from natural sources (AO, 2001). Although biogenic isoprene is not considered a pollutant and does not directly pose a health hazard, in urban areas, it can react with nitrogen oxides (NOx) to produce tropospheric ozone, which is harmful to health and contributes to air pollution (Millet et al., 2016). Moreover, its interaction with hydroxyl radicals indirectly prolongs the atmospheric lifetime of methane, thereby intensifying global warming (Fehsenfeld et al., 1992; Sharkey et al., 2008).

Various ecosystems, including freshwater, marine, soil, and the phyllosphere, have been identified as isoprene sinks. Soil microorganisms exhibit a remarkable capability to degrade volatile compounds (Chen et al., 2013; Sorkhoh et al., 2011), including biogenic volatile organic compounds (BVOCs) (Albers et al., 2018). One study shows that isoprene was the most absorbed compound in soil among other isoprenoids (Mu et al., 2023), which are the most abundant BVOCs, with an estimated annual consumption of approximately 20.4 teragrams (Tg) (Cleveland and Yavitt, 1998, 1997). Soil microbial communities play a key role in isoprene degradation. Recent studies suggest that isoprene

* Corresponding author.
E-mail addresses: toungporn.u@cmu.ac.th (T. Uttarotai), tjmcgen@essex.ac.uk (T.J. McGenity), sawannee.sut@kmutt.ac.th (S. Sutheeworapong), wuttichai.mhu@biotec.or.th (W. Mhuantong), nuttapon.k@cmu.ac.th (N. Khongdee), sakunnee.b@cmu.ac.th (S. Bovonsombut), thararat.chitov@cmu.ac.th (T. Chitov).

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consumption rates vary significantly across different ecosystems. For instance, oil palm soil harbors diverse isoprene-degrading bacteria, indicating a potentially larger isoprene sink than previously thought (Carrión et al., 2020). Additionally, microbial activity can consume up to 68 % of atmospheric isoprene in certain environments, depending on local conditions (Gray et al., 2015).

Diverse isoprene-degrading bacterial genera, including those belonging to phyla Actinomycetota (formerly known as Actinobacteria) and Pseudomonadota (formerly known as Proteobacteria), have been identified (Acuña Alvarez et al., 2009; El Khawand et al., 2016; Gibson and Larke-Mej, 2020; Johnston et al., 2017). The degradation mechanisms of isoprene in some bacteria, particularly in *Variovorax* and many members of Actinomycetota, such as *Arthrobacter, Nocardia*, and *Rhodococcus*, have been described (Rix et al., 2023; Sims et al., 2023; Dawson et al., 2022; van Hylckama Vlieg et al., 2000; Crombie et al., 2015; Cleveland and Yavitt, 1997). It appears that the key to the typical degradation process is the isoprene monooxygenase enzyme, production of which is induced by isoprene itself (El Khawand et al., 2016). Genetic studies of various isoprene-degrading bacteria have revealed a high level of genetic conservation around the *iso* gene cluster. This cluster comprises genes *isoABCDEF* that encode a multi-subunit isoprene monooxygenase and genes *isoGHIJ* that encode glutathione transferase and enzymes involved in subsequent stages of isoprene oxidation (Dawson et al., 2023; van Hylckama Vlieg et al., 2000, 1999, 1998). The *isoA* gene, encoding the putative active site of isoprene monooxygenase, is believed to play a crucial role in isoprene biodegradation, thus extensively used as a molecular target for identifying isoprene degraders (Dawson et al., 2020; Carrión et al., 2018; Johnston et al., 2017; El Khawand et al., 2016).

Many forest tree species emit large amounts of isoprene, and since forests are among the largest biomes of the biosphere, this dominant biogenic compound can significantly contribute to the hydrocarbon exchange between the biosphere and the atmosphere (Sharkey et al., 2008). In this study, we investigated isoprene biodegradation in a highland tropical forest. Highland tropical forest plays an important role in the climate system but is one of the most endangered types of forest and very vulnerable to climate change. The highland tropical forest chosen as the site of study adopts a sustainable economic crop cultivation model that promotes forest conservation, having coffee and tea as the main economic crops planted among the forest trees.

Coffee (*Coffea* spp.) and tea (*Camellia sinensis*) are among the most widely cultivated perennial crops in tropical and subtropical regions, particularly in agroforestry systems where they are integrated with native or plantation forests (Moreira et al., 2020; Hajiboland, 2017). Although these crops are not known as major isoprene emitters, their associated soil microbes may play an important role in isoprene degradation. The extent of isoprene emissions from coffee and tea plants remains unclear, as most studies on it have focused on forest tree species (Jardine et al., 2020). Most research on isoprene-degrading bacteria has emphasized natural forest soils, particularly in temperate ecosystems, with studies identifying key bacterial taxa involved in isoprene metabolism (Larke-Mejía et al., 2019; Crombie et al., 2018). However, studies on isoprene-degrading bacteria in tropical agroforestry systems remain limited. Investigating this process in agroforestry settings is important, as these systems integrate economic crops with native vegetation, potentially influencing microbial community composition and function. Assessing microbial isoprene degradation in these environments will contribute to a better understanding of their ecological role and potential significance in atmospheric hydrocarbon cycling.

In this study, we hypothesize that, regardless of whether coffee and tea plants release isoprene, their associated soils harbor active communities of isoprene-degrading bacteria. Our objectives are to (1) assess the potential of soil microbes associated with coffee and tea plants in this agroforestry setting for isoprene degradation; (2) isolate and identify isoprene-degrading bacteria and determine their degradation activity; and (3) determine the mechanism of degradation of isoprene by the bacterial isolates through genome sequence analysis.

2. Materials and methods

2.1. Soil sampling and analysis of soil physicochemical properties

In this study, soil samples (100 g each) were collected from the top 3-cm surface beneath the plants of coffee (*Coffea arabica* L.) and tea (*Camellia sinensis* L. Kuntze var. *assamica* (Mast.) Kitam), planted among forest trees. The selected sites are in Mae Kampong Village, located in the mountainous part (approximately 1300 m above sea level) of Chiang Mai, north of Thailand (18°51′56.7″N, 99°21′01.0″E). For each plant species, five replicate soil samples were collected from beneath different plants, with each sample taken 1 m apart using autoclaved spoons and sterile bags. The sampling was carried out in September and January, which were in the rainy and dry seasons, respectively. These represent the period of two distinct climatic conditions of Northern Thailand.

The pH of the soil samples was measured using a pH meter, following the protocol of a 1:1 solid to water ratio. The evaluation of soil organic matter was conducted using the Walkley-Black method (Walkley and Black, 1934). Total nitrogen content was determined through the micro-Kjeldahl technique (Ma and Zuazaga, 1942). For the assessment of available phosphorus, the molybdate blue method was utilized (Murphy and Riley, 1962), following the Bray II extraction protocol (Bray and Kurtz, 1945). Exchangeable potassium was measured using atomic absorption spectrometry, in conjunction with ammonium acetate extraction at pH 7.0. Soil moisture content was ascertained through the standard oven drying technique. Following these assessments, the properties of the soil collected beneath coffee and tea plants during the dry and rainy seasons were compared. Statistical analyses were performed using a one-way ANOVA and post-hoc Tukey's HSD tests to identify significant differences.

2.2. Soil sample preparation for isoprene degradation analysis

Soil samples were prepared into 1/10 dilutions in minimal medium (contained per liter: 0.5 g NaCl, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, 1 g NH_4NO_3, 1.1 g Na_2HPO_4, 0.25 g KH_2PO_4, 50 mg Cycloheximide, 10 mg $FeSO_4{\cdot}7H_2O$, 0.64 mg $Na_2EDTA{\cdot}3H_2O$, 0.1 mg $ZnCl_2$, 0.015 mg H_3BO_3, 0.175 mg $CoCl_2{\cdot}6H_2O$, 0.15 mg $Na_2MoO_4{\cdot}2H_2O$, 0.02 mg $MnCl_2{\cdot}4H_2O$, 0.01 mg $NiCl_2{\cdot}6H_2O$, 0.05 mg *p*-Aminobenzoic acid, 0.02 mg Folic acid, 0.02 mg Biotin, 0.05 mg Nicotinic acid, 0.05 mg Calcium pantothenate, 0.05 mg Riboflavin, 0.05 mg Thiamine HCl, 0.1 mg Pyridoxine HCl, 0.001 mg Cyanocobalamin, and 0.05 mg Thioctic acid (Uttarotai et al., 2021)). These sample preparations were then placed in glass 125 cm^3 vials, which were sealed with PTFE/silicone septa to ensure a gastight environment. Isoprene (supplied by Sigma-Aldrich, St. Louis, MO, USA) was injected into each vial to achieve a concentration of 7.2×10^5 parts per billion by volume (ppbv) (Uttarotai et al., 2021). To account for the non-microbial loss of isoprene, the control vials containing autoclaved soil beneath the five coffee and tea plants were included in each batch. The treatment and control vials were incubated for 96 h at 27 °C.

2.3. Measurement of isoprene degradation by soil microbes

The remaining isoprene in each vial (prepared in Section 2.2) was measured every 24 h for 96 h. Headspace gas samples (100 μL) were collected through the septum and directly injected into an Agilent 7890B Gas Chromatography-Flame Ionization Detection (GC-FID) system. The GC-FID was equipped with an Agilent J&W HP-5 column (320 μm × 30 m) with a stationary phase thickness of 0.25 μm. The injector and the column temperatures were 250 °C and 80 °C, respectively. The detector temperature was 275 °C. Helium, used as the carrier gas, was supplied at 1.5 mL min^{-1}. The analysis was conducted over 96 h. For statistical analysis, an ANOVA test was performed using Jamovi (v. 1.2).

2.4. Recovery of isoprene-degrading bacteria from soil

The soil samples incubated with isoprene for 96 h were subjected to bacterial isolation. Ten-fold serial dilutions were prepared, and a 0.1 mL portion of each dilution was spread on minimal medium agar (composition listed in Uttarotai et al., 2021, with 1.5 % agar). The agar plates were then incubated for 7 days in the presence of isoprene (1 mL (specification as above) in a 2.5 L airtight container). Representatives of bacterial colonies were picked based on colony morphology. The isolates were identified through 16S rRNA sequencing. Genomic DNA samples were extracted using a GF-1 Bacterial DNA Extraction Kit (Vivantis, Shah Alam, Malaysia). PCR was performed using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGT TACGACTT-3′) (Weisburg et al., 1991) and MyTaq Mix (Bioline, London, UK). The PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles consisting of denaturation at 95 °C for 15 s, primer annealing at 55 °C for 15 s, and strand extension at 72 °C for 30 s. A final elongation step was performed at 72 °C for 5 min. The amplified products were purified using the GF-1 PCR Clean-up Kit (Vivantis, Shah Alam, Malaysia). The Sanger sequences were subjected to trimming and concatenation using Chromas (v.2.6.6) and BioEdit (v.7.0.5.3) and blasted against the NCBI 16S rRNA database using BlastN.

2.5. Determination of the presence of isoA

The DNA samples from the selected isolates (prepared as above, 2.4) were subjected to the detection of gene *isoA* using PCR analysis. Two sets of primers were employed: one yielding PCR products of 1015 bp (El Khawand et al., 2016) and the other of 497 bp (Carrión et al., 2018). Both PCRs used MyTaq Mix (Bioline, London, UK).

2.6. Measurement of isoprene degradation by bacterial isolates

The pure cultures of the bacterial isolates retrieved from minimal medium were examined for their levels of isoprene degradation. A loopful of each culture was first resuspended in 100 μl of minimal medium. It was then transferred to a 125 cm^3 vial containing 9.9 mL of minimum medium and sealed with a PTFE/silicone cap. Isoprene was injected (7.2×10^5 ppbv, as described in Section 2.2) through the cap into each vial. The vials containing minimal medium without isoprene were also included and served as controls. The treatments and controls were prepared in five replicates.

Every day, bacterial growth was assessed through OD_{600} using a Jenway 7300 Spectrophotometer. In parallel, the remaining isoprene was determined using a GC-FID (as described in Section 2.3). After 7 days of incubation, the bacterial cultures were drawn for spread-plating on minimal medium agar and further incubated in a 2.5 L sealed jar containing 1 mL of 99 % liquid isoprene for an additional 5 days to ensure the purity of the isoprene-degrading bacterial isolates.

2.7. Genomic DNA preparation for genome analysis

The genomic DNA obtained from selected bacterial isolates that could utilize isoprene (Section 2.6) was used for genome analysis. DNA was extracted from the pure cultures using a GF-1 Bacterial DNA Extraction Kit (as described in Section 2.4). Subsequently, the DNA samples were subjected to agarose gel electrophoresis to verify their quality. Quantification of the DNA was performed using the Quant-iT PicoGreen dsDNA assay (Thermo Fisher Scientific, Waltham, MA, USA) on a Nanodrop spectrophotometer (NanoDrop 3300, Thermo Scientific, Wilmington, DE, USA).

2.8. Genome sequencing and assembly

DNA libraries for genome sequencing were prepared by Macrogen, Inc. (Seoul, Korea). A paired-end 350-bp insert-size library was created using the TruSeq Nano DNA kit (San Diego, California, USA). Subsequently, the libraries underwent sequencing on the Illumina NovaSeq6000 platform, employing 2 × 151 bp paired-end sequencing according to standard Illumina operating protocols. To assess the quality of the raw reads, FastQC v. 0.11.9 (Joshi and Fass, 2011) was employed for assessing the quality of raw sequences. Trimmomatic (v. 0.30) (Bolger et al., 2014) was applied to remove any remaining adapter sequences, using a sliding window quality cutoff of Q15. For the *de novo* genome assembly, SPAdes version 3.7 (Bankevich et al., 2012) was utilized with default parameters.

Table 1
Physicochemical properties of soil samples under coffee and tea plants during dry and rainy seasons.

Soil property	Soil beneath coffee plants		Soil beneath tea plants	
	Dry season	**Rainy season**	**Dry season**	**Rainy season**
Soil moisture (% dry weight)	39.10 ± 0.63 [b]	44.05 ± 0.42 [a]	39.24 ± 0.58 [b]	43.97 ± 0.34 [a]
pH	5.85 ± 0.09 [a]	5.69 ± 0.05 [a]	5.49 ± 0.07 [b]	5.11 ± 0.05 [c]
Organic matter (%)	5.18 ± 0.01 [a]	5.16 ± 0.01 [a]	4.25 ± 0.08 [b]	3.86 ± 0.09 [c]
Total nitrogen (%)	0.34 ± 0.01 [a]	0.34 ± 0.01 [a]	0.33 ± 0.01 [a]	0.22 ± 0.01 [b]
Available phosphorus ($mg \cdot kg^{-1}$)	11.86 ± 0.12 [c]	14.42 ± 0.08 [b]	15.46 ± 0.20 [b]	16.90 ± 0.26 [a]
Exchangeable potassium ($mg \cdot kg^{-1}$)	221.81 ± 5.79 [c]	223.28 ± 2.33 [c]	288.41 ± 3.48 [b]	316.86 ± 8.48 [a]

Note: Values are shown as means ± SE ($n = 5$). Different letters (a–c) denote significant statistical differences ($p < 0.05$) compared within the row, analyzed using the Tukey's HSD test.

2.9. Phylogenomic characterization and average nucleotide identity (ANI) analysis

The genome sequences of the three bacterial isolates were subjected to phylogenomic analysis, together with selected publicly available genomes of the closest species, which were initially identified through BlastN analysis of the 16S rRNA gene. These identified species served as a foundation to guide the selection of genomes for the subsequent phylogenomic investigation. The Pathosystems Resource Integration Centre (PATRIC) (Wattam et al., 2014) was utilized as the platform for constructing a codon-based tree based on 500 single-copy genes present in all genomes studied. The gene sequences were aligned using MAFFT version 7.397 (Nakamura et al., 2018). To construct the tree, the maximum likelihood method with the Jones-Taylor-Thornton (JTT) model was applied, utilizing RAxML version 8.2.11 (Stamatakis, 2014). Branch support values were determined through 100 replicates of fast bootstrapping.

Additionally, the similarities among the genomes of closely related bacterial isolates were examined by calculating Average Nucleotide Identity (ANI) values using Orthologous Average Nucleotide Identity Tool (OAT) version 0.93.1 (Lee et al., 2016). The resulting values were then visualized with Seaborn libraries (Waskom, 2021) in Python.

2.10. Genome annotation and analysis of amino acid sequence similarities

To assess the quality of the assembly, QUAST version 5.0.2 (Mikheenko et al., 2018) was employed, and sequences below 200 bp in length were excluded prior to the annotation process. The completeness of the genome assembly was determined using Benchmarking Universal Single-Copy Orthologs (BUSCO) version 4.1.3 (Manni et al., 2021). Subsequently, annotation was carried out using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 5.2, incorporating the

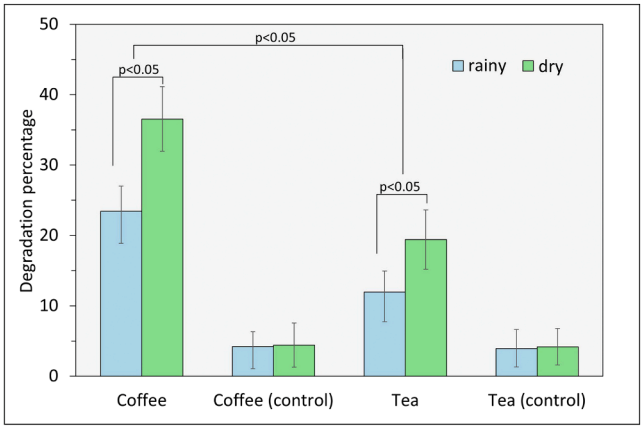

Fig. 1. Percentage degradation of isoprene in microcosms containing soils from underneath coffee and tea plants, collected in rainy and dry seasons, in minimal medium with isoprene (7.2×10^5 ppbv) incubated for 96 h. Controls are autoclaved soils. Error bars show ± SE ($n = 5$).

best-placed reference protein set and GeneMarkS-2+ methods.

The amino acid sequences of the proteins encoded by the genes in the *iso* cluster were compared with those of the known isoprene-degrading strains of the same genus using the web-based BlastP version 2.12.0.

3. Results and discussion

3.1. Soil physicochemical properties beneath coffee and tea plants

The physicochemical properties (including soil moisture, pH, organic matter, total nitrogen, available phosphorus, and exchangeable potassium) of soil samples collected in two seasons beneath the coffee and tea plants were compared (Table 1). Generally, the soils had high moisture contents (above 39 % dry weight in both distinct seasons), which are as expected for the type and location of the forest (an evergreen tropical forest located at a high altitude, *ca.* 1300 m above mean sea level). The soils were slightly acidic, with pH ranging from 5.11 to 5.85, nutrient-rich, and had high levels of organic matter (OM > 3 %), which are typical characteristics of tropical forest soils. These factors suitably accommodate coffee and tea planting in this integrative crop-in-forest plantation.

As for the seasonal comparison of soil parameters, the soil moisture contents from both sources were higher in the rainy season than in the dry season, as expected. There were no significant differences in chemical and nutrient parameters for soil beneath coffee plants between the two seasons, except for available phosphorus, which was significantly higher in the rainy season ($p < 0.05$). For soil beneath tea plants, the chemical and nutrient values were statistically different ($p < 0.05$) between the two seasons, and they were different from those of the coffee-plant soil except for the total nitrogen in the dry-season soil. It is interesting to note that the soils beneath tea plants had the highest level of exchangeable potassium among all groups, despite their lower levels of pH and organic matter.

3.2. Isoprene degradation by soil microbes associated with coffee and tea plants

The degradation of isoprene by soil microbes associated with coffee and tea plants in this agroforestry plantation was investigated. The experimental design involved comparing the degradation in the rainy and dry seasons. The outcomes of these experiments, as presented in Fig. 1, showed microbial degradation of isoprene. The autoclaved soil, which served as the control, exhibited minimal isoprene loss, ranging from 3.92 % to 4.42 %. In contrast, non-autoclaved soils demonstrated significantly higher losses, which were between 11.95 % and 36.54 %.

Table 2
Correlation coefficient (r) from Pearson correlation analysis between soil properties and isoprene degradation.

Soil parameter	Coffee (Dry)	Coffee (Rainy)	Tea (Dry)	Tea (Rainy)
pH	0.36 (p = 0.55)	−0.73 (p = 0.16)	0.92 (p = 0.02)*	0.06 (p = 0.92)
Organic matter	0.60 (p = 0.28)	−0.58 (p = 0.30)	0.35 (p = 0.56)	0.31 (p = 0.61)
Total nitrogen	0.28 (p = 0.63)	−0.40 (p = 0.50)	0.57 (p = 0.31)	0.23 (p = 0.72)
Available phosphorus	0.40 (p = 0.50)	−0.35 (p = 0.57)	0.64 (p = 0.24)	0.29 (p = 0.64)
Exchangeable potassium	0.42 (p = 0.48)	0.92 (p = 0.02)*	0.51 (p = 0.38)	0.38 (p = 0.49)
Soil moisture	0.20 (p = 0.72)	0.91 (p = 0.03)*	0.44 (p = 0.46)	0.22 (p = 0.73)

Note: (*) indicates statistically significant correlations ($p < 0.05$).

This data confirms that the degradation observed in the experimental microcosms was primarily due to microbial activities rather than non-microbiological processes, underscoring the significance of the microbial biodegradation of isoprene in these soils.

These results clearly indicated that soils beneath coffee and tea plants in this agroforestry form of plantation contained isoprene-degrading microbes and confirmed a widespread distribution of isoprene-degrading microorganisms in tropical forest soils, a finding consistent with previous observations with other soil types. Carrión et al. (2020) found that microbial isoprene degradation occurred in tropical oil palm plantation soils, identifying diverse isoprene-degrading bacteria involved in this process. In temperate ecosystems, Gray et al. (2015) quantified microbial isoprene degradation in soils, showing that microbial activity can remove up to 68 % of atmospheric isoprene and identifying key microbial taxa (*Actinobacteria, Proteobacteria, Gemmatimonadetes*, and *Zygomycota*). These findings reinforce the concept that microbial degradation of isoprene is a widespread process across different ecosystems, though its efficiency may be shaped by climate, land use, and plant-associated microbial diversity.

Fig. 1 also shows that the percentages of soil isoprene degradation varied by associated plants and seasons. Significant differences in degradation percentages were observed between the soils underneath coffee and tea plants (Anova/Tukey's HSD test; $p < 0.05$). These results could be attributed to the microbial diversity in soils associated with various plant species and the isoprene degradation levels, as found in some previous works (Carrión et al., 2018; Uttarotai et al., 2021). Additionally, differences in isoprene degradation were observed across seasons. The percentages of degradation in soils collected in the dry season were statistically significantly higher (Anova/Tukey's HSD test; $p < 0.05$) than those in the rainy season for both plant types (36.54 % compared to 23.45 % for soils beneath coffee plants and 19.39 % compared to 11.95 % for soils beneath tea plants).

To further investigate the relationships between soil physicochemical properties and microbial isoprene degradation, Pearson's correlation analysis was conducted separately for soils beneath coffee and tea plants in both seasons (Table 2).

The analysis revealed that in coffee-rainy soils, exchangeable potassium ($r = 0.92$, $p = 0.02$) and soil moisture ($r = 0.91$, $p = 0.03$) were significantly correlated with degradation, suggesting that these factors may enhance microbial activity in high-moisture environments. While potassium is not directly linked to isoprene metabolism, it plays a role in microbial osmoregulation and enzyme activation (Stautz et al., 2021). The strong correlation with soil moisture further suggests that water availability may facilitate microbial activity, either by improving substrate diffusion or supporting microbial proliferation (Butcher et al., 2020).

In tea-dry soils, pH exhibited a strong positive correlation with isoprene degradation ($r = 0.92$, $p = 0.02$), suggesting that soil acidity

Table 3
Identification of isoprene-degrading bacterial isolates using 16S rRNA gene sequencing.

Isolate	Source	Cell morphology	Colony morphology	Closet relative	Identity (%)
C3V	Soil beneath coffee plants	Gram-positive, rod-shaped	circular, pulvinate, pale orange-colored colonies with entire edge	*Rhodococcus qingshengii* strain djl62 (NCBI accession no. NR043535)	100.0
T2V	Soil beneath tea plants	Gram-positive, rod-shaped	circular, convex, cream-colored colonies with entire edge	*Rhodococcus wratislaviensis* strain DSM 44107 (NCBI accession no. NR118605)	100.0
N1V	Soil beneath tea plants	Gram-positive, rod-shaped	circular, pulvinate, pale orange-colored colonies with entire edge	*Gordonia polyisoprenivorans* strain Kd2 (NCBI accession no. NR171465)	99.6

may influence microbial degradation of isoprene under drier conditions. This observation aligns with previous findings that demonstrate pH as a key regulator of microbial community composition, affecting the abundance of bacterial taxa, including Actinomycetota and Proteobacteria, which play crucial roles in isoprene metabolism (Sridhar et al., 2022).

In contrast, no significant correlations were found in tea-rainy soils. This suggests that additional factors beyond the measured soil properties may regulate microbial isoprene degradation under wetter conditions. Furthermore, tea plants are known to release secondary metabolites that could selectively influence microbial activity, which might explain the weaker dependence on soil physicochemistry observed here (Zhang et al., 2021). It should be noted that the laboratory conditions, although beneficial for standardizing the experiment, may not fully represent the natural environments that influence microbial dynamics. Further research, especially *in situ* isoprene degradation measurement, is imperative to validate these findings in natural settings. Moreover, a detailed analysis encompassing the diversity of microbial communities could offer a deeper insight into the mechanisms governing isoprene degradation in these environments.

3.3. Soil-derived bacteria capable of isoprene degradation

Three distinct isolates of isoprene-degrading bacteria were recovered from isoprene-enriched soil samples from beneath coffee and tea plants. They exhibited distinguishing morphological characteristics (Table 3). The 16S rRNA gene sequences of these isolates have been deposited in the NCBI database under accession numbers OQ619000, OQ619013, and OQ619016. Based on 16S rRNA sequences, one isolate (C3V) obtained from coffee plant soil had the most similarity to *Rhodococcus qingshengii*. Two isolates from tea plant soil were more closely related to *Rhodococcus wratislaviensis* and *Gordonia polyisoprenivorans*.

Rhodococcus and *Gordonia* belong to the phylum Actinomycetota and have been well-recognized for their potential roles in the biodegradation of hydrocarbons, including isoprene (Johnston et al., 2017; van Hylckama Vlieg et al., 1998). Researchers have explored the genome sequences of these bacteria and provided comprehensive insights into their isoprene degradation mechanisms (Larke-Mejía et al., 2020; Crombie et al., 2015). This study, however, marks the first discovery of these isoprene-degrading bacterial genera from tropical soils, expanding our understanding of their ecological distribution and their potentials in bioremediation of this climate-active gas in the tropical environment. We then proceeded with the investigation of their ability to degrade isoprene and the genetic characteristics related to this property.

3.4. Presence of isoA and isoprene degradation by Rhodococcus and Gordonia isolates

Many isoprene-degrading bacterial strains harbor *isoA*, the gene encoding a subunit of isoprene monooxygenase, which is believed to play a crucial role in isoprene degradation (Crombie et al., 2015). Here, we examined the presence of *isoA* in the *Rhodococcus* and *Gordonia* isolates using two sets of PCR primers specific for this gene (El Khawand et al., 2016; Carrión et al., 2018). Both sets of primers were able to detect the gene in all three isolates. This suggests that the genes in both species

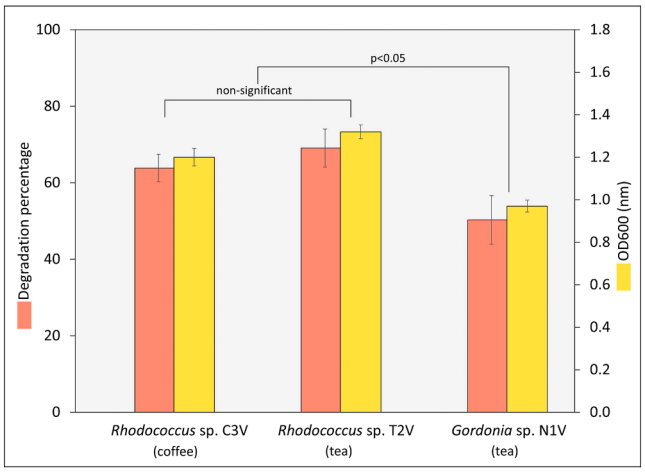

Fig. 2. Percentages of isoprene degradation by isolates of *Rhodococcus* and *Gordonia* from tropical forest soils associated with coffee and tea plants and their corresponding growth, expressed as OD_{600} values, after seven days of incubation in minimal medium with isoprene (7.2×10^5 ppbv). Error bars show $\pm$ SE ($n = 5$).

resemble those in the known isoprene-degrading strains.

Based on these results, we evaluated the ability of these bacterial isolates to utilize isoprene as the sole source of carbon and energy over a seven-day incubation period (Fig. 2). Alongside this, we assessed their growth through OD_{600} measurements. *Rhodococcus* sp. T2V had the highest isoprene degradation level of 69.07 % and exhibited the highest OD_{600} of 1.32. *Rhodococcus* sp. C3V had a degradation level of 63.82 % and grew to an OD_{600} of 1.20. The differences in degradation and growth between these two *Rhodococcus* isolates were not significant ($p > 0.05$).

Gordonia sp. N1V had an isoprene degradation level of 50.28 %, which was significantly lower than the degradation levels of the *Rhodococcus* isolates ($p < 0.05$). The results indicate the inherent differences in isoprene catabolic activities among different bacterial genera. We also observed this in our previous study (Uttarotai et al., 2021).

Compared with other bacterial genera outside Actinomycetota, such as an *Alcaligenes* strain 13f retrieved from a tropical forest soil (Uttarotai et al., 2022), the *Rhodococcus* and *Gordonia* isolates obtained in this study still had higher isoprene-degrading abilities. In the experiment conducted under the same condition but over a longer period of incubation with isoprene (18 days), *Alcaligenes* sp. 13f displayed a degradation level of only 32.6 %. Notably, it did not harbor the *isoA* gene. This suggested that the isoprene-degrading abilities of the *Rhodococcus* and *Gordonia* isolates obtained in this study may be linked to the activities of isoprene monooxygenase, a multi-subunit enzyme encoded by the *iso* cluster (Crombie et al., 2015). The *iso* cluster, including *isoA*, has been primarily identified in isoprene-degrading bacteria, particularly within phyla Actinomycetota (*Rhodococcus* and *Gordonia*) and Proteobacteria (*Variovorax* and *Sphingopyxis*), which metabolize isoprene as a carbon source (Larke-Mejía et al., 2019; Crombie et al., 2018). The presence of this gene cluster is strongly associated with high isoprene degradation potential, as non-isoprene-degrading strains typically lack these genes

Table 4
Genomic profiles of isoprene-degrading *Rhodococcus* and *Gordonia* isolates analyzed in this study.

Genomic information	*Rhodococcus* sp. C3V	*Rhodococcus* sp. T2V	*Gordonia* sp. N1V
Total length (bp)	6,848,786	11,763,595	6,205,491
GC content (%)	62.31	67.05	66.94
Number of contigs	100	575	131
N50 (bp)	424,508	92,149	217,304
CDS (total)	6330	10,982	5631
Genes (coding)	6264	10,576	5535
Genes (RNA)	60	56	55
rRNAs (5S, 16S, 23S)	1, 1, 1	1, 1, 1	1, 1, 1
tRNAs	54	49	49
ncRNAs	3	3	3
Pseudogenes (total)	66	406	96

(Carrión et al., 2018). However, while *isoA* serves as a key genetic marker, its presence alone does not confirm active isoprene metabolism without functional validation (Dawson et al., 2020). Additionally, some bacteria, such as *Alcaligenes* sp. 13f, have been reported to degrade isoprene despite lacking *isoA* (Uttarotai et al., 2022), suggesting that alternative pathways may exist. To further explore the genetic basis of isoprene metabolism in these isolates, we conducted whole-genome characterization.

3.5. Overall genome characteristics of Rhodococcus and Gordonia isolates

The whole-genome sequences of *Rhodococcus* C3V, *Rhodococcus* T2V, and *Gordonia* N1V have been deposited in the NCBI database under BioProject PRJNA940297. Their genomes were sequenced and assembled, and their key genomic features are listed in Table 4.

The length of the *Rhodococcus* sp. C3V genome, which was determined to be approximately 6.85 Mbp (Table 4), is typical for the *Rhodococcus* genus. The genome of *Rhodococcus* sp. T2V, although much larger than that of *Rhodococcus* sp. C3V, is still within the size range for *Rhodococcus* genomes, which can vary from 3.89 to 12.41 Mbp. The GC contents of both isolates (62.31 to 67.05 %) are also within the typical range of *Rhodococcus* previously reported by Ying et al. (2019), which was 61.67 to 70.67 %. The differences in the genomic profiles of the two *Rhodococcus* isolates suggested that they do not belong to the same species. Likewise, the genome of *Gordonia* sp. N1V, which was 6.20 Mbp in length with a GC content of 66.94 %, is similar to that of the isoprene-degrading *Gordonia* sp. i37, which possesses a genome of 6.23 Mbp and a GC content of 66.8 % (Johnston et al., 2017).

3.6. Phylogenomic and comparative analyses of isoprene-degrading bacterial isolates

A phylogenomic analysis was conducted on the three isoprene-degrading bacterial isolates. The closely related strains, according to the 16S rRNA gene sequences, and the known isoprene-degrading strains were also taken into consideration when the detailed phylogenomic analysis was undertaken (see Section 2.9). The genome sequences of the three isolates were compared with those of the previously identified strains using the PATRIC workspace (Wattam et al., 2014). Our findings, shown in Fig. 3, revealed distinct affiliations for each isolate. *Rhodococcus* isolates C3V and T2V showed close associations with *Rhodococcus baikonurensis* JCM 18801 and *Rhodococcus koreensis* DSM 44498, respectively. *Gordonia* sp. N1V closely resembles the isoprene degrader *Gordonia* sp. i37, and both are related to the type strain

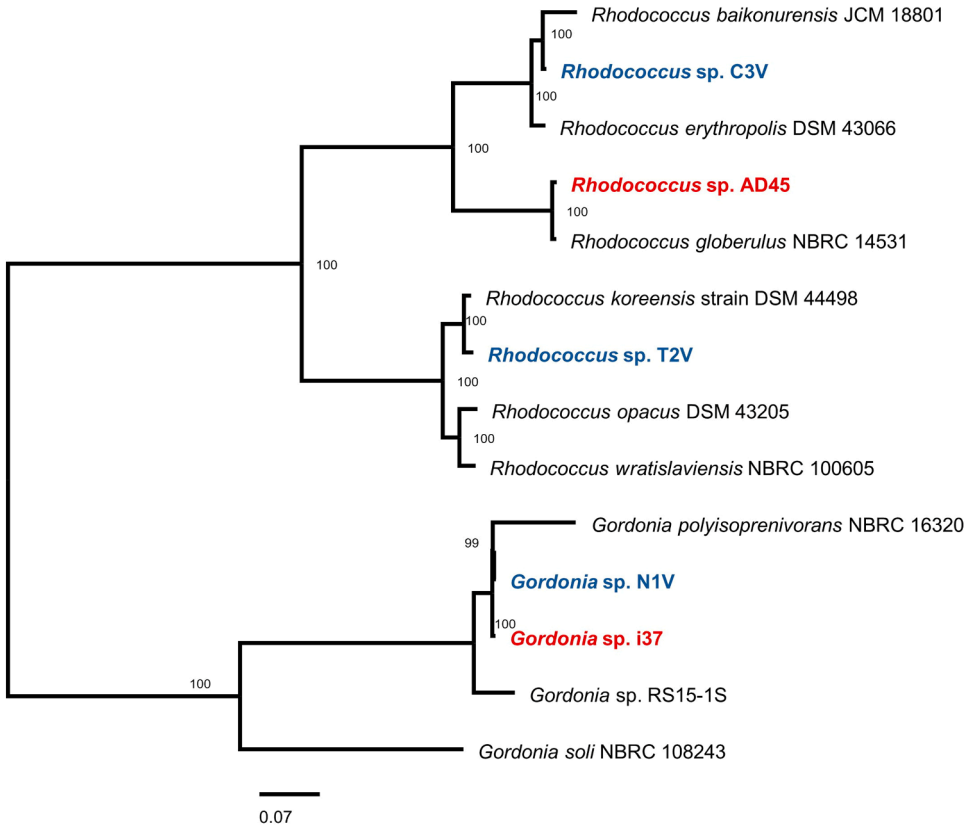

Fig. 3. Phylogenomic analysis of the isoprene-degrading isolates obtained in this study and the related strains, based on a codon-based tree constructed from 500 single-copy genes present in all genomes studied. The *Rhodococcus* and *Gordonia* isolates from this study (blue) are shown with the degrees of their relatedness to their closely related counterparts (black) and known isoprene-degrading strains (red).

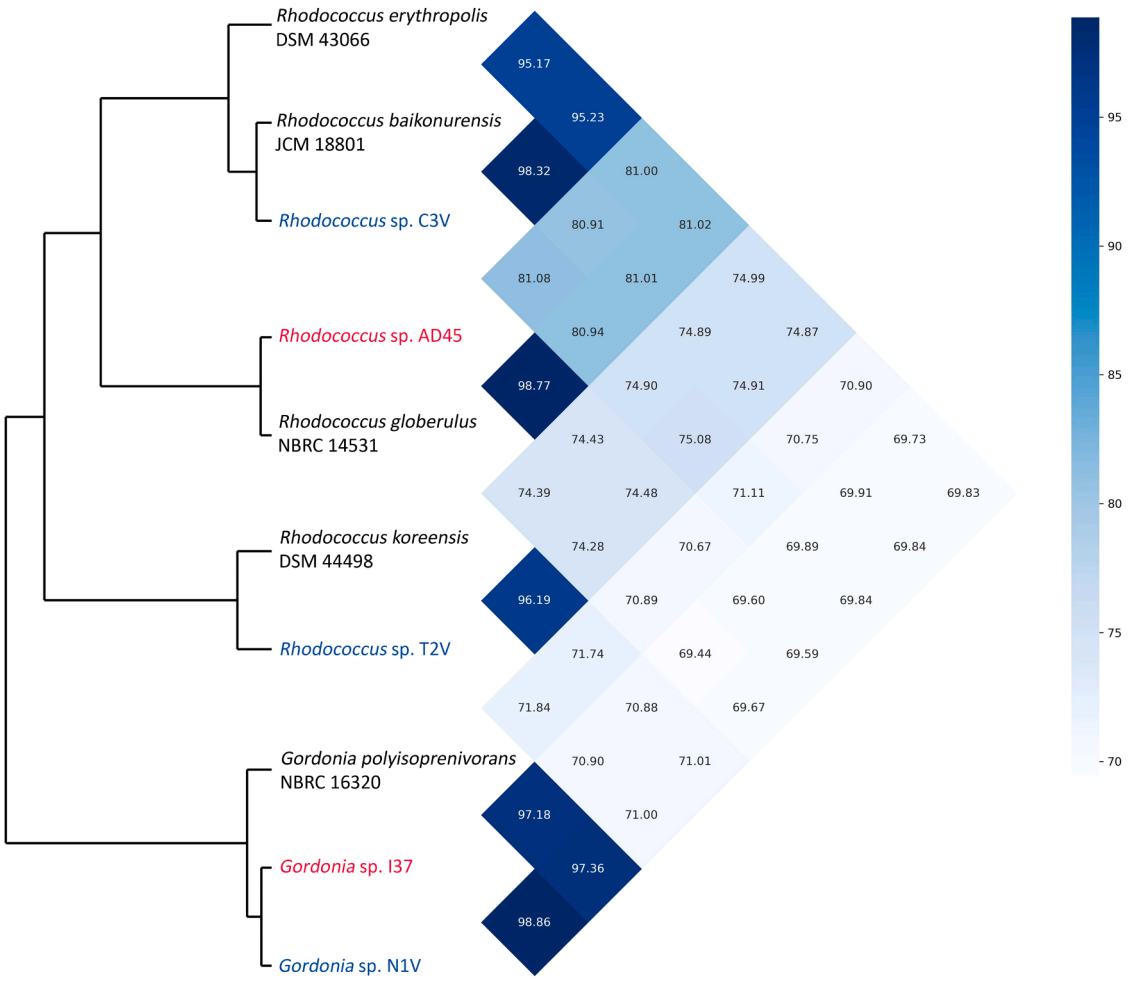

Fig. 4. Average Nucleotide Identity (ANI) heatmap illustrating ANI values between the isolates obtained in this study (shown in blue) and their closely related strains (shown in black or red). The known isoprene-degrading strains included in this analysis are shown in red. The degrees of similarity are indicated by the numbers and the color intensity.

Gordonia polyisoprenivorans NBRC 16320. This suggests a potential functional similarity in isoprene degradation between *Gordonia* sp. N1V and *Gordonia* sp. i37.

To complement the phylogenomic analysis, Average Nucleotide Identity (ANI) analysis was conducted to confirm the species assignments based on genome comparisons, as illustrated in Fig. 4. The ANI values, calculated against five closely related bacterial strains and two known isoprene-degrading strains, revealed that the three isolates had ANI values above the 95 % threshold, indicating shared species identity (Goris et al., 2007; Konstantinidis and Tiedje, 2005). Specifically, *Rhodococcus* sp. C3V aligned with *Rhodococcus baikonurensis* JCM 18801 with an ANI value of 98.32 %, and *Rhodococcus* sp. T2V with *Rhodococcus koreensis* DSM 44498 with an ANI value of 96.19 %. *Gordonia* sp. N1V exhibited a very close relationship to *Gordonia* sp. i37, with an ANI value of 98.86 %, and to *Gordonia polyisoprenivorans* NBRC 16320, with an ANI value of 97.36 %. The ANI analysis results align well with the phylogenomic analysis, further validating the species identification.

3.7. Isoprene metabolic gene clusters in Gordonia and Rhodococcus

The comparative genomic analysis of genes responsible for isoprene degradation, as depicted in Fig. 5A, demonstrates a notable similarity in gene organization between *Gordonia* sp. N1V and the previously characterized *Gordonia* sp. i37, a known isoprene degrader (Johnston et al., 2017). Similarly, *Rhodococcus* C3V and *Rhodococcus* T2V exhibit similar gene arrangements to isoprene-degrading *Rhodococcus* sp. AD45 (Johnston et al., 2017). In all bacterial isolates from this study (N1V, C3V, and T2V), a complete *iso* cluster (*isoABCDEF*) was identified. The genes in this cluster encode oxygenase subunits (IsoABE), a reductase (IsoF), a ferredoxin (IsoC), and a coupling protein (IsoD) that form the isoprene monooxygenase complex (van Hylckama Vlieg et al., 2000, 1999, 1998). The presence of a complete cluster with similar gene organization is indicative of the functional capability for isoprene degradation. Notably, the gene *isoGHIJ*, located upstream of the isoprene monooxygenase complex, was consistently present across all isolates. The subsequent steps of the isoprene degradation pathway implicate a putative coenzyme A transferase, a dehydrogenase, and two glutathione transferases encoded by these genes (van Hylckama Vlieg et al., 2000, 1999, 1998). The conserved genetic architecture of the *iso* cluster may explain the efficiency of the *Rhodococcus* and *Gordonia* isolates in isoprene degradation.

Next, the enzymes associated with isoprene degradation within the genomes of the isoprene-degrading isolates were analyzed for their amino acid sequence similarities. The proteins encoded by the genes in the *iso* cluster (*isoA* through *G*) were compared with those of the reference strains (*Gordonia* i37 and *Rhodococcus* AD45) within the same genus. The analysis revealed high similarities in the amino acid sequences of the Iso proteins between each bacterial isolate and the reference strain, with all identities exceeding 75 %. Notably, for the *Gordonia* isolate N1V, several sequences, including IsoA, IsoB, IsoC, IsoF, IsoI, and IsoJ, were identical to *Gordonia* sp. i37. The IsoD, IsoE, and IsoG sequences were also highly similar. For *Rhodococcus* C3V and T2V,

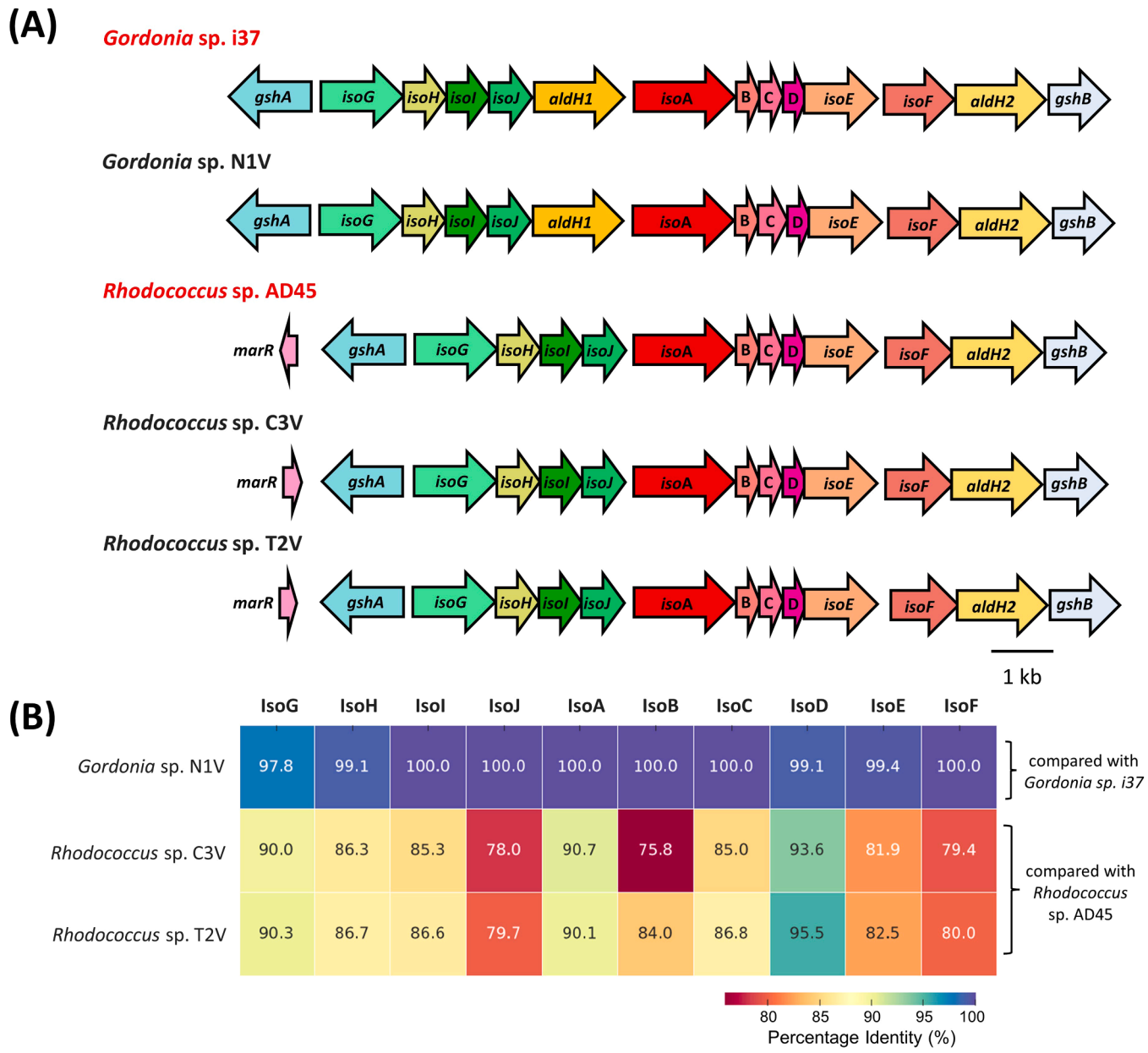

Fig. 5. Comparative analysis of gene clusters related to isoprene degradation and the encoded protein sequences in the *Gordonia* and *Rhodococcus* isolates: **(A)** Gene organization in *Gordonia* sp. N1V compared with *Gordonia* sp. i37 and in *Rhodococcus* sp. C3V and T2V compared with *Rhodococcus* sp. AD45; **(B)** Sequence similarities of the proteins involved in isoprene degradation, analyzed using BlastP version 2.12.0. The figure in each block is the percentage identity of the amino acid sequences of each isolate, compared with the reference strain of the same genus. The degrees of similarity are also demonstrated by colors.

high percentage identities were also observed between the Iso sequences of each of these isolates and those of *Rhodococcus* AD45. The conserved *iso* gene cluster and amino acid sequences of proteins involved in isoprene metabolism underscore the functional significance of these genomic regions in the biodegradation of isoprene by these widely distributed genera of Actinomycetota.

4. Conclusions

In this study, the degradation of isoprene by soil microbes associated with coffee and tea plants grown in a tropical evergreen forest was explored, and the isoprene-degrading bacteria were isolated and characterized. We found that, despite the non-isoprene-producing nature of the crops, the soil beneath them harbored isoprene-degrading bacteria. The degradation levels varied by the associated plant species and seasons. From the soil beneath coffee and tea plants, isoprene-degrading bacteria were isolated: two *Rhodococcus* and one *Gordonia* species. The bacterial isolates were capable of utilizing isoprene as their sole carbon and energy source, although the *Rhodococcus* isolates showed higher growth than *Gordonia*. Genetic analysis confirmed the presence of the *isoA* gene, which encodes isoprene monooxygenase α-subunit, a key enzyme in isoprene degradation, in all three isolates. However, further functional validation is needed to confirm its expression and activity. The isolates are closely related to *Rhodococcus baikonurensis* JCM 18801, *Rhodococcus koreensis* DSM 44498, and *Gordonia polyisoprenivorans* NBRC 16320, according to the ANI analysis of the genome sequence applied in this study. The finding of these isoprene-degrading genera of Actinomycetota in association with coffee and tea plants in this forest plantation contributes to our understanding of their geographical distribution, their potential role in the cycling of isoprene, and their potential positive impact on climate in such evergreen tropical forest biomes.

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Data availability statement

The 16S rRNA sequence and the genomic data are available on the NCBI database (accession number OQ619000, OQ619013, OQ619016 and PRJNA940297).

CRediT authorship contribution statement

Toungporn Uttarotai: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Terry J. McGenity:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Sawannee Sutheeworapong:** Formal analysis. **Wuttichai Mhuantong:** Formal analysis. **Nuttapon Khongdee:** Formal analysis, Writing – review & editing. **Sakunnee Bovonsombut:** Conceptualization, Funding acquisition, Supervision. **Thararat Chitov:** Conceptualization, Funding acquisition, Project administration, Methodology, Investigation, Resources, Supervision, Data curation, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

All data is publicly available.

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