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Functional profiling of *Syzygium aromaticum*-associated bacteria reveals PGPR traits for tropical biofertilizer development

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Abstract. *Kesaulya H, Hidayat W, Simarmata R, Patty J. 2025. Functional profiling of* Syzygium aromaticum*-associated bacteria reveals PGPR traits for tropical biofertilizer development. Biodiversitas 26: 6130-6139. Syzygium aromaticum* (clove) is a tropical spice of major economic and ecological importance in Indonesia, yet the microbial communities associated with its cultivation remain poorly characterized. Harnessing native Plant Growth-Promoting Rhizobacteria (PGPR) offers a sustainable alternative to chemical inputs and supports resilient agroecosystems. This study explored the biofertilizer potential of bacterial strains isolated from *S. aromaticum*-growing regions in Central Maluku, Indonesia. Rhizospheric and endophytic isolates were characterized using physiological assays and 16S rRNA sequencing to assess key PGPR traits, including auxin production, phosphate solubilization, nitrogen fixation, and enzymatic activities relevant to nutrient cycling and stress tolerance. Eleven isolates were obtained, with greater diversity from the rhizosphere (9 isolates) than from leaves (2 isolates). Screening demonstrated multiple plant-beneficial traits, including indole-3-acetic acid production, phosphate solubilization, and growth in nitrogen-free medium, complemented by hydrolytic enzyme activities and catalase-based stress tolerance. Three isolates showed particularly strong multifunctional profiles: T5RCHM21 (high IAA, phosphate solubilization, nitrogen-fixation trait, broad pH tolerance), T6RCHM3 (highest phosphate solubilization and cellulolytic activity), and T4RCHM3 (very strong catalase with additional enzymatic indices). 16S rRNA analysis grouped isolates mainly within *Bacillus*, with *Brevibacterium*, and *Klebsiella*, supporting phenotype-driven selection and future field validation for practical application.

Keywords: Biofertilizer, bioinoculant, endophyte, rhizosphere, *Syzygium aromaticum*

INTRODUCTION

Endophytic and rhizospheric bacteria play a significant role in enhancing plant health and ecological adaptation, particularly in tropical environments. These microbes, collectively known as Plant Growth-Promoting Rhizobacteria (PGPR), are increasingly recognized for their role in sustainable crop production (Compant et al. 2019; Rivera-Hernández et al. 2024; Zeng et al. 2025). Endophytic bacteria are increasingly recognized as integral components of the plant holobiont that regulate host stress responses and promote sustainable crop production under variable climatic conditions (Afzal et al. 2019; Ansabayeva et al. 2025).

Globally, PGPR-based biofertilizers are considered environmentally sustainable alternatives to synthetic agricultural inputs. These microbial inoculants enhance nutrient bioavailability, improve soil physicochemical properties, and maintain microbial balance, thereby contributing to long-term soil fertility, particularly in tropical agroecosystems (Backer et al. 2018; Dubey et al. 2025). However, most current research focuses on annual staple crops such as rice, maize, and legumes (Trivedi et al. 2020), whereas studies on perennial tropical spice crops remain limited.

Syzygium aromaticum (L.) Merr. & L.M.Perry) (clove), a perennial spice native to the Maluku Islands of Indonesia, holds significant economic, cultural, and ecological importance. Indonesia is the world's largest producer and exporter of cloves, which are widely utilized in the food, pharmaceutical, and fragrance industries. Nevertheless, the sustainability of clove cultivation is increasingly constrained by soil nutrient depletion, recurrent disease outbreaks such as dieback caused by *Valsa eugeniae,* and a reduction in soil microbial diversity due to prolonged chemical fertiliser application (Sha et al. 2023). These challenges highlight the need for alternative and eco-friendly strategies to sustain soil fertility and crop productivity through the utilization of indigenous microbial resources.

Endophytic and rhizospheric PGPR offer promising biological solutions to these constraints. Their functional traits support nutrient cycling and plant growth, making them promising candidates for the development of biofertilizers. In tropical soils, phosphorus is abundant yet largely unavailable due to fixation by iron and aluminium oxides. Microorganisms capable of secreting organic acids can solubilize these bound forms, thereby improving phosphorus uptake and nutrient-use efficiency in clove plants (Vassileva et al. 2020; Ling et al. 2022). Likewise, biological nitrogen fixation mediated by the nifH gene

provides a renewable nitrogen source, reducing dependency on synthetic fertilizers (Kumar and Dubey 2020).

The ecological adaptation of endophytic and rhizospheric bacteria associated with tropical perennial crops presents a unique opportunity for biofertilizer development. Indigenous strains often exhibit adaptive traits such as tolerance to high temperature, acidity, salinity, and local pathogens that enhance their ecological fitness and functional stability under critical field conditions (Eid et al. 2021; Khan et al. 2022b). Despite Indonesia's remarkable microbial diversity, the functional and taxonomic characterization of native bacteria associated with *S. aromaticum* remains poorly understood. Previous studies have been largely limited to preliminary isolation or morphological identification, with little emphasis on physiological characterization or molecular taxonomy (Ishak et al. 2018; Dewi Kirana et al. 2025).

This study aimed to address this knowledge gap by conducting a systematic integrative characterization of endophytic and rhizospheric bacterial isolates from *S. aromaticum* plants in Central Maluku, Indonesia. The research integrated comprehensive physiological assays, enzymatic activity evaluations, and 16S rRNA-based taxonomic identification to assess their potential as reliable plant growth-promoting rhizobacteria. The functional traits examined include, Indole-3-Acetic Acid (IAA) synthesis, phosphate solubilization, nitrogen fixation, and hydrolytic enzyme activities related to nutrient cycling and stress tolerance.

This study provides a comprehensive profiling of native PGPR associated with *S. aromaticum* in Indonesia, linking their functional diversity to ecological adaptation. The findings are expected to support the development of sustainable, region-specific biofertilizers that enhance nutrient efficiency, minimize dependence on chemical fertilizers, and promote the sustainable cultivation of tropical spice crops within the biodiversity-rich ecosystems.

MATERIALS AND METHODS

Study site and sampling design

The study was conducted in Mamala Village, Central Maluku, Indonesia, at an elevation of 23.4 m above sea level (a.s.l.). Twelve healthy *Syzygium aromaticum* trees were purposively selected and categorized into two age groups: young (≤5 years; n = 6) and mature (≥10 years; n = 6). Tree age was initially determined through structured interviews with experienced local farmers and subsequently validated using morphological characteristics based on established regional cultivation guidelines.

Sample collection

Sampling was executed for the analysis of microbial communities in the rhizosphere soil and leaf tissues of *S. aromaticum*. The specific sampling protocols adhered to the methodologies described by Contreras-Cornejo et al. (2025) and Pednekar et al. (2025).

Bacterial isolation and purification

Plant tissue-derived isolates were processed using the triple streaking method, a sequential streaking technique on agar media designed to yield single colonies while minimizing cross-contamination. Colonies with uniform morphological characteristics were selected as candidate isolates and repeatedly sub-cultured until stable, homogeneous cultures were established, ensuring reliability for downstream analyses. The purified isolates were preserved in a 10% glycerol solution and stored at -20°C, with glycerol serving as a cryoprotectant to prevent cellular damage caused by ice crystal formation, thereby maintaining microbial viability during long-term storage. This preservation strategy allows the isolates to be reused for diverse applications, including physiological and biochemical characterization, enzymatic profiling, bioactivity assays, and biomolecule extraction. The overall isolation procedure followed the protocols of Tripathi et al. (2025), who developed a TRIzol-based method for the simultaneous extraction of RNA, DNA, and proteins, and Pednekar et al. (2025), who emphasized endophytic microbial isolation through rigorous surface sterilization of plant tissues and selective culture techniques. Integrating these approaches not only ensured the viability of microbial isolates but also enabled parallel molecular analyses of plant tissues, thereby providing a comprehensive framework for understanding plant biosystems and their interactions with endophytic microorganisms.

Screening of PGPR traits

Indole-3-Acetic Acid (IAA) production

The Indole-3-Acetic Acid (IAA) quantification was performed using a colorimetric assay based on Salkowski's reagent, following the method described by Dey et al. (2004). Bacterial isolates were grown in L-tryptophan-supplemented liquid medium to enhance IAA biosynthesis, and cell-free supernatants were obtained following phase separation. The chromogenic reaction that developed after the addition of Salkowski's reagent was measured spectrophotometrically at 530 nm. IAA concentrations were determined using a calibration curve prepared with analytical-grade IAA standards.

Phosphate solubilization

Phosphate solubilization was assessed on Pikovskaya agar by measuring halo zones around colonies. Halo zone measurements were subsequently used to calculate the Phosphate Solubilization Index (PSI). PSI was calculated by taking the ratio of the total diameter (colony diameter + halo zone) and the colony diameter (mm) of the isolated petri plates (Patel et al. 2022; Babu et al. 2025).

Nitrogenase activity

Nitrogen fixation activity was evaluated using semi-solid nitrogen-free NfB medium, specifically formulated to support the growth of diazotrophic bacteria. The medium composition, consisting of malic acid as the primary carbon source and essential mineral salts, was prepared according to established protocols widely employed in the characterization of nitrogen-fixing microorganisms (Baldani

et al. 2014). Bacterial isolates were inoculated into the medium following standard procedures for assessing nitrogen fixation capacity, as outlined by Glick (2012), and incubated under controlled conditions. The evaluation was based on characteristic diazotrophic growth indicators in NfB medium, including the formation of a subsurface pellicle and a color change to blue, which reflects alkalinization associated with nitrogen fixation activity (Baldani et al. 2014). Isolates exhibiting positive responses were re-examined to ensure consistency and reproducibility. All analytical procedures followed validated methodologies that have been extensively applied in diazotroph research (Glick 2012; Baldani et al. 2014).

Enzymatic activity assays

The hydrolytic enzyme activities of amylase, cellulase, and protease were evaluated using selective agar plate assays. Each bacterial isolate was inoculated onto specific media and incubated under optimal conditions to facilitate the formation of clear hydrolysis zones. Following incubation, enzymatic activity was quantified using the Enzyme Activity Index (EAI), calculated using the following formula:

$$\text{EAI} = \frac{\text{Diameter of clear zone} - \text{Colony diameter}}{\text{Colony diameter}}$$

This ratio-based metric reflects the extent of substrate degradation relative to colony size and is widely employed in microbiological research to assess extracellular enzyme production by PGPR (Ferbiyanto et al. 2015; Miłek and Lamkiewicz 2022). All measurements were conducted in triplicate.

Catalase activity was assessed qualitatively by applying hydrogen peroxide (H_2O_2) directly to bacterial colonies and observing the resulting effervescence. The intensity of bubble formation was scored using a semi-quantitative scale ranging from 1 (weak) to 4 (very strong), following the protocols described by Murali and Patel (2017) and Yuan et al. (2021).

Molecular identification

Genomic DNA was extracted, and the 16S rRNA gene was amplified and sequenced for taxonomic identification. Sequence alignment and phylogenetic analysis were performed using standard bioinformatics tools. These identification and analysis procedures referenced the methodologies described by Packeiser et al. (2013) and Tamura et al. (2021).

Phylogenetic analysis

The 16S rRNA sequences were aligned using the ClustalW program. Phylogenetic relationships were subsequently inferred using the Neighbor-Joining method. This analysis implemented the Kimura 2-parameter substitution model. The entire phylogenetic analysis was performed using the MEGA 11 software (Tamura et al. 2021).

Data analysis

All physiological and enzymatic data were analyzed descriptively and presented as mean±Standard Deviation (SD). To mitigate the risk of pseudoreplication, individual measurements were averaged at tree level and subsequently categorized based on isolate origin (rhizosphere vs. endophyte) and tree age category. Functional assays were performed on eleven bacterial isolates (n = 11). To ensure reproducibility and statistical power, each isolate was tested using ten independent biological replicates (n = 10). Nitrogenase activity was analyzed using Chi-square tests, while catalase activity was interpreted semi-quantitatively based on scores assigned to the level of foam formation.

RESULTS AND DISCUSSION

Initial isolation and diversity of bacterial communities

A total of eleven morphologically distinct bacterial isolates were successfully obtained from the endophytic and rhizospheric compartments of *Syzygium aromaticum* collected in Mamala Village, Central Maluku, Indonesia. The distribution of these isolates indicated rhizosphere dominance; two isolates originated from leaf tissues (endophytes), while nine isolates were recovered from the rhizosphere soil (Table 1). Significant morphological variation (pigmentation, colony shape, and elevation) indicated substantial taxonomic diversity. The rhizosphere clearly exhibited greater microbial richness and heterogeneity compared to the endosphere. This finding is consistent with literature defining the rhizosphere as a more metabolically dynamic microenvironment (Compant et al. 2010; Ling et al. 2022).

Physiological characterization of PGPR traits

Among the eleven bacterial isolates examined, three strains exhibited notable multifunctionality and ecological significance for tropical bioinoculant candidates. T5RCHM21 demonstrated the highest levels of IAA production and phosphate solubilization, coupled with active nitrogenase expression and tolerance to alkaline conditions (up to pH 10) (Table 2). These attributes suggest strong potential for enhancing root development and nutrient mobilization in nutrient-depleted tropical soils. T6RCHM3 recorded the highest Phosphate Solubilization Index (PSI = 4.778) and robust cellulolytic activity, indicating its capacity to facilitate phosphorus release and accelerate organic matter decomposition. T4RCHM3 exhibited exceptional catalase activity, alongside measurable amylase and protease functions, underscoring its role in mitigating oxidative stress and promoting plant resilience under abiotic stress conditions. Collectively, these three isolates represent complementary functional traits: phytohormone biosynthesis, nutrient mobilization, and stress tolerance that are essential for the formulation of consortium-based bioinoculants adapted to tropical agroecosystems.

Table 1. Initial isolation and diversity of bacterial communities associated with *Syzygium aromaticum*

Isolate	Source	Morphological variation		
		Pigmentation	Colony shape	Elevation
CHMD6	Leaf endophyte	White	Circular	Raised
CHMA10	Leaf endophyte	White	Circular	Crateriform
T1RCHM20	Rhizosphere (mature plant)	White	Circular	Flat
T1RCHM27	Rhizosphere (mature plant)	Cream	Circular	Flat
T4RCHM3	Rhizosphere (mature plant)	White	Circular	Convex
T4RCHM10	Rhizosphere (mature plant)	White	Circular	Crateriform
T5RCHM16	Rhizosphere (young plant)	Orange	Circular	Raised
T5RCHM21	Rhizosphere (young plant)	Yellow	Circular	Convex
T6RCHM3	Rhizosphere (young plant)	White	Circular	Convex
T6RCHM5	Rhizosphere (mature plant)	White	Rhizoid	Flat
T1RCHM15	Rhizosphere (mature plant)	White	Circular	Convex

Table 2. Physiological traits of endophytic and rhizospheric bacterial isolates associated with *Syzygium aromaticum*

Isolate	IAA (ppm)	Phosphate Solubilization Index	Nitrogen fixation (pH)
CHMD6	0.420±0.02	Not detected	9
CHMA10	0.988±0.03	0.758±0.02	9
T1RCHM20	0.157±0.01	0.267±0.01	9
T1RCHM27	0.166±0.01	0.708±0.02	9
T4RCHM3	0.103±0.01	0.235±0.01	9
T4RCHM10	0.129±0.01	0.659±0.02	9
T5RCHM16	0.420±0.02	Not detected	9
T5RCHM21	0.988±0.03	0.758±0.02	10
T6RCHM3	0.376±0.02	4.778±0.05	9
T6RCHM5	0.157±0.01	0.267±0.01	9
T1RCHM15	0.376±0.02	4.778±0.05	9

Enzymatic profiling

The enzymatic characterization of *S. aromaticum*-associated PGPR isolates revealed a broad spectrum of hydrolytic and antioxidative activities that are ecologically relevant to nutrient cycling and stress mitigation in tropical soils. Elevated amylase and cellulase activities, particularly in isolates T1RCHM27 and T6RCHM3 (Table 3), indicate a strong capacity for organic matter decomposition and enhanced carbon turnover in the rhizosphere, which supports microbial persistence and nutrient bioavailability (de Andrade et al. 2023; Ishaq et al. 2025). Although protease activity was comparatively lower, its presence contributes to the mineralization of organic nitrogen sources, complementing the nitrogen-fixing capabilities of other strains (Timofeeva et al. 2023). High catalase activity, as observed in T4RCHM3 and T1RCHM15, reflects robust oxidative stress tolerance, a trait essential for microbial survival under abiotic stressors such as drought, salinity, and reactive oxygen species accumulation (Shabaan et al. 2022; Saeed et al. 2023). These enzymatic traits not only enhance microbial ecological fitness but also contribute to plant resilience by facilitating nutrient mobilization and protecting host tissues from oxidative damage. Collectively, the enzyme-based functional profiles support the strategic selection of complementary strains for consortium-based bioinoculant candidates, integrating nutrient cycling efficiency with environmental stress tolerance (Jing et al. 2023).

16S rRNA gene amplification and sequencing analysis

The amplification of the 16S rRNA gene was successfully performed on all eleven bacterial isolates (endophytic and rhizospheric). Visualization of the Polymerase Chain Reaction (PCR) products on 1% agarose gel revealed a clear and consistent single band at approximately 1500 base pairs (bp) (Figure 1). This result definitively validates the successful amplification and confirms the quality and readiness of the DNA samples for further sequencing procedures (Baer et al. 2024).

Molecular sequencing analysis demonstrated primary taxonomic diversity among the isolates, distributed within the phyla Firmicutes and Proteobacteria. Most isolates exhibited a strong affiliation with the genus *Bacillus* (encompassing species such as *Bacillus cereus*, *Bacillus aerius*, and *Bacillus pseudomycoides*), in addition to the genera *Brevibacterium* and *Klebsiella* (Table 4). The dominance of the genus *Bacillus* in the rhizosphere was confirmed, a finding consistent with existing literature that underscores its extensive ecological adaptation (Rasul et al. 2024; de Alcântara Neto et al. 2025). Species identification accuracy was further supported by the high nucleotide similarity (≥99%) observed in most isolates (eight of eleven) when compared to GenBank reference sequences.

Despite the high degree of taxonomic similarity, substantial functional variation was observed in the isolates' physiological and enzymatic characteristics (as detailed in Tables 2 and 3). For instance, closely related isolates frequently demonstrated significant differences in their phosphate solubilization capacity or IAA production. This strain-level functional divergence has critical implications, affirming that the selection criteria for biofertilizer formulations must be based on measurable functional capabilities, rather than solely on taxonomic identity (Ünlü et al. 2024). A function-centric approach is highly recommended, given that the genus *Bacillus*, despite its taxonomic uniformity, is known to possess broad metabolic diversity. This metabolic diversity consequently serves as the primary determinant of PGPR effectiveness in field applications (Jang et al. 2023).

Table 3. Enzymatic profiles of endophytic and rhizospheric bacterial isolates associated with *Syzygium aromaticum*

Isolate	Amylolytic Index	Cellulolytic Index	Proteolytic Index	Catalase (score)
CHMD6	Not detected	0.500±0.01	0.200±0.01	2.0±0.2 (++)
CHMA10	1.800±0.04	0.677±0.02	0.333±0.01	3.0±0.1 (+++)
T1RCHM20	1.455±0.03	3.000±0.05	0.444±0.02	2.0±0.2 (++)
T1RCHM27	3.000±0.03	5.000±0.04	0.412±0.02	3.0±0.1 (+++)
T4RCHM3	0.467±0.01	0.188±0.01	0.200±0.01	4.0±0.1 (++++)
T4RCHM10	0.583±0.02	0.571±0.02	0.182±0.01	1.0±0.1 (+)
T5RCHM16	0.429±0.01	0.571±0.02	0.182±0.01	1.0±0.1 (+)
T5RCHM21	0.429±0.01	0.571±0.02	0.182±0.01	3.0±0.1 (+++)
T6RCHM3	Not detected	7.000±0.05	Not detected	2.0±0.2 (++)
T6RCHM5	Not detected	Not detected	Not detected	1.0±0.1 (+)
T1RCHM15	Not detected	Not detected	Not detected	4.0±0.1 (++++)

Note: Catalase score (+): weak, (++): moderate, (+++): strong, (++++): very strong

Phylogenetic analysis based on 16S rRNA

The phylogenetic tree (Figure 2), constructed using the Maximum Likelihood (ML) method, visualizes the clustering of *S. aromaticum* PGPR isolates into five major clades. The tree structure clearly reflects the sequence divergence and evolutionary relationships among the isolates, marked by high bootstrap support within the *Bacillus* group. The distribution of isolates across these distinct phylogenetic groups validates the essential role of these taxa in tropical agroecosystems. Overall, these findings reinforce the taxonomic identities previously determined through initial molecular sequencing.

The distinct phylogenetic positions of the *Brevibacillus* and *Klebsiella* isolates highlight the taxonomic diversity underlying the functional differentiation observed in the biochemical and enzymatic assays. Concurrently, the minimal genetic distance among the *Brevibacterium* isolates suggests a very close strain affiliation, possibly even an identical strain identity.

Moderate bootstrap support (e.g., 53%) at certain nodes indicates limitations in phylogenetic resolution. This phenomenon is common in soil microbial communities and may be attributed to mechanisms such as horizontal gene transfer or cryptic speciation. To ensure more robust and accurate classification in the future, it is recommended that higher-resolution approaches, such as Multi-Locus Sequence Typing or whole-genome sequencing, be implemented.

Despite the high degree of taxonomic similarity at the species level (≥99% identity) and their clustering within the same groups (Figure 2), significant functional variation was observed in the isolates' physiological and enzymatic characteristics (Table 2, Table 3). This strain-level (intra-species) functional divergence represents a noteworthy observation with implications for biofertilizer candidate selection.

Consequently, the integration of phylogenetic and functional data definitively demonstrates that the selection of isolates for biofertilizer formulation must be based on measurable functional capabilities and ecological adaptation, rather than solely on taxonomic identity. This analysis provides a crucial genetic map to guide the development of adaptive, tropical ecology-based biofertilizers through the optimal utilization of *S. aromaticum* microbial diversity.

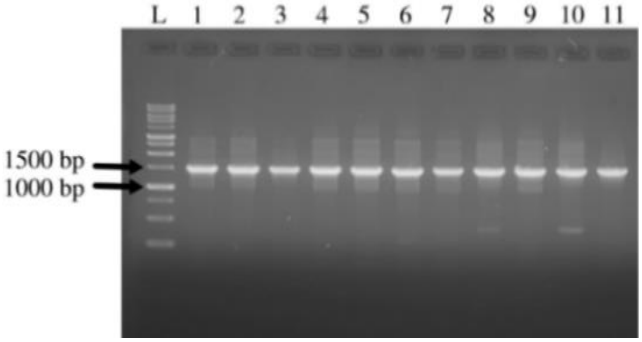

Figure 1. Amplification profiles of representative *S. aromaticum*-associated bacterial isolates. Lanes 1-10 represent individual isolates, while M denotes the molecular size marker (1 kb ladder). Clear bands at ~1500 bp confirm successful amplification of the 16S rRNA gene

Table 4. 16S rRNA gene sequence similarity of endophytic and rhizospheric bacterial isolates associated with *S. aromaticum*

Isolate	Species	Homology (%)
CHMD6	*Bacillus* sp.	99.24%
CHMA10	*Bacillus* sp.	98.00%
T1RCHM20	*Bacillus cereus*	99.50%
T1RCHM27	*Bacillus aerius*	99.59%
T4RCHM3	*Bacillus pseudomycoides*	99.31%
T4RCHM10	*Bacillus* sp.	99.03%
T5RCHM16	*Brevibacterium* sp.	99.22%
T5RCHM21	*Brevibacterium* sp.	98.67%
T6RCHM3	*Klebsiella pneumoniae*	99.02%
T6RCHM5	*Bacillus halotolerans*	98.89%
T1RCHM15	*Bacterium* sp.	99.09%

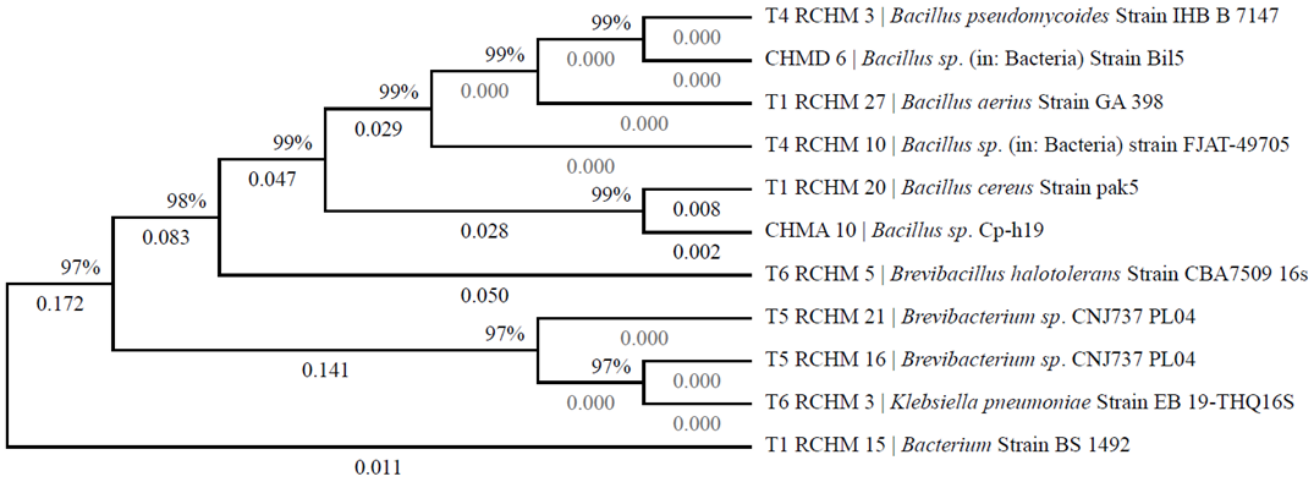

Figure 2. Phylogenetic relationships of *S. aromaticum*-associated bacterial isolates. The phylogenetic tree was constructed using MEGA 11 software based on 16S rRNA sequences generated in this study. Maximum Likelihood analysis with 1000 bootstrap replications was applied, with branch lengths indicating sequence divergence and bootstrap values representing clade support. Isolates clustered within the genera *Bacillus*, *Brevibacterium*, *Klebsiella*, *Brevibacillus*, and *Bacterium*. The scale bar corresponds to 0.02 substitutions per site

Discussion

Initial isolation and diversity of bacterial communities

This study presents the first comprehensive functional profiling of *S. aromaticum*-associated PGPR from Central Maluku, Indonesia, emphasizing their diverse physiological and enzymatic characteristics and their ecological adaptation to tropical soils. These results provide a robust foundation for the development of region-specific biofertilizers to enhance nutrient efficiency and support sustainable clove cultivation. Eleven bacterial isolates were successfully recovered from rhizospheric soil and leaf tissues, with rhizospheric strains exhibiting greater abundance and functional versatility. These isolates demonstrated key PGPR traits, including IAA biosynthesis, phosphate solubilization, nitrogen fixation, and hydrolytic enzyme activities, indicating ecological adaptation to tropical soil conditions. Several strains exhibited multifunctionality and tolerance to alkaline environments, reinforcing their potential as bioinoculant candidates. The observed niche-specific functional differentiation between endophytic and rhizospheric isolates reflects an ecological division of labor, with implications for the strategic design of microbial consortia tailored to clove cultivation. These findings address a critical gap in tropical microbiome research and provide a foundation for developing region-specific biofertilizers to support sustainable spice production.

Physiological characterization of PGPR traits

The primary traits identified included IAA production, phosphate solubilization, and nitrogen fixation, each contributing to nutrient efficiency and plant resilience in tropical agroecosystems (Backer et al. 2018; Shah et al. 2021). IAA production varied among isolates, with CHMA10 and T5RCHM21 demonstrating the highest levels. These strains, affiliated with *Bacillus* and *Brevibacterium*, are well known for their auxin biosynthetic capacity (Abo Elsoud et al. 2023; Ganesh et al. 2024). High IAA levels support early root development and plant vigor (Rafique et al. 2024; Sierra-García et al. 2024). Furthermore, auxin-mediated signaling enhances microbe-plant interactions and contributes to adaptive stress responses (Aloo et al. 2022; Deng et al. 2024; Han 2024).

Phosphate solubilization was observed in most isolates, with T6RCHM3 and T1RCHM15 producing the largest halo zones. These traits are particularly advantageous in nutrient-limited tropical soils (Aliyat et al. 2022; Pang et al. 2024). Phosphate-solubilizing strains improve nutrient availability in clove cultivation. Nitrogen fixation was confirmed through pellicle formation and a color change in NfB medium, indicating diazotrophic activity. Notably, T5RCHM21 exhibited tolerance up to pH 10, suggesting physiological adaptation to the alkaline soil conditions prevalent in Maluku (Eid et al. 2021; Khan et al. 2022a). The co-expression of nitrogen fixation and phosphate solubilization in multifunctional strains such as CHMA10 and T6RCHM3 reinforces their suitability for consortium-based bioinoculant candidates (Timofeeva et al. 2023; Joshi et al. 2025).

The quantitative evidence (Tables 2 and 3) demonstrates that *S. aromaticum*-associated PGPR isolates are functionally active and encompass a broad spectrum of plant growth-promoting mechanisms. The functional differentiation observed between endophytic and rhizospheric isolates illustrates an ecological division of labor (Santoyo et al. 2016). Endophytic strains primarily contribute to the synthesis of IAA, whereas rhizospheric strains are more effective in nutrient mobilization, particularly phosphate solubilization and nitrogen fixation (Han 2024). This complementary distribution of traits underscores the rationale for a consortium-based strategy that integrates diverse microbial functions to enhance clove productivity in tropical ecosystems.

Enzymatic profiling and functional implications for biofertilizer development

The enzymatic profile highlighted key roles in nutrient cycling and stress adaptation. Isolate T1RCHM27 exhibited strong amylase activity and cellulase activity, facilitating the degradation of complex substrates and improving carbon availability. T4RCHM3 and T1RCHM15 exhibited strong catalase activity, a critical mechanism for coping with oxidative stress. Although protease activity was comparatively low, it nonetheless contributed to the mineralization of organic nitrogen. These enzymatic traits enhance microbial resilience in tropical soils and support plant tolerance under adverse environmental conditions.

Strains outside the *Bacillus* clade, such as T5RCHM16 (*Brevibacterium* sp.), T6RCHM3 (*Klebsiella pneumoniae*), and T1RCHM15 (*Bacterium* sp.), formed distinct phylogenetic branches and exhibited specialized functional profiles. T6RCHM3 demonstrated exceptional phosphate solubilization and cellulolytic activity, reflecting adaptation to tropical soils rich in organic matter but limited in available nutrients. Meanwhile, T1RCHM15, despite its unresolved taxonomic classification, displayed strong oxidative stress tolerance and phosphate mobilization, underscoring its potential as a strategic component in microbial consortium design.

The taxonomic classification of isolate T1RCHM15 could not be resolved beyond the designation *Bacterium* sp., despite exhibiting a high sequence similarity (>99%). This limitation is likely attributable to the restricted discriminatory capacity of partial 16S rRNA sequences and the absence of closely related reference strains in available databases. Achieving finer resolution would require Multi-Locus Sequence Analysis (MLSA) or whole-genome sequencing. Accordingly, in this study, T1RCHM15 is consistently reported as *Bacterium* sp., with emphasis placed on its functional characteristics as the primary basis for consortium selection.

These findings highlight the importance of phenotype-based selection, emphasizing actual functional capacity and ecological compatibility over taxonomic affiliation in bioinoculant candidates. The alignment between functional performance (Table 2, Table 3) and phylogenetic structure (Figure 2) provides a robust framework for assembling metabolically synergistic and ecologically stable PGPR consortia. This approach is essential for developing clove-specific biofertilizers that are resilient to tropical agroecosystem constraints and aligned with sustainable agricultural practices.

The functional diversity exhibited by *S. aromaticum*-associated PGPR isolates provides a solid foundation for designing microbial consortia tailored to tropical agricultural systems. Preliminary compatibility assays demonstrated that strains with complementary traits, such as phosphate solubilization, nitrogen fixation, and antioxidative enzyme activity, can be co-cultured without antagonistic interactions. This functional synergy is particularly relevant in tropical agroecosystems, where nutrient limitations and abiotic stressors frequently co-occur. The inclusion of multifunctional and alkali-tolerant strains, such as T5RCHM21, T6RCHM3, and T4RCHM3, enhances the ecological resilience and nutrient mobilization potential of the proposed consortium.

Although several isolates were taxonomically assigned to the genus *Bacillus,* including CHMD6, CHMA10, T1RCHM20, T1RCHM27, T4RCHM3, and T4RCHM10, they exhibited notable functional divergence despite close phylogenetic relationships. For example, T1RCHM27 showed strong amylolytic and cellulolytic activities, while T4RCHM10 displayed only moderate responses. Catalase activity also varied, with T4RCHM3 scoring 4.0 and T6RCHM5 only 1.0. These discrepancies suggest that taxonomic proximity does not guarantee functional equivalence, as strain-specific genomic adaptations and microhabitat pressures may drive divergence. Therefore, phenotype-based selection should be prioritized in bioinoculant candidates to ensure ecological compatibility, functional complementarity, and field-level efficacy. Future research should prioritize field validation under diverse agroclimatic conditions and explore carrier materials capable of maintaining microbial viability and activity. Collectively, this study presents a practical framework for advancing clove-specific biofertilizer technologies aligned with sustainable agriculture goals in tropical regions.

This study confirms that bacterial isolates, both endophytic and rhizospheric, sourced from *S. aromaticum*, possess significant diversity in physiological and enzymatic properties highly relevant for biofertilizer formulation. This variation is interpreted as evidence of ecological adaptation to specific microbial niches, which, in turn, contributes to a synergistic potential for supporting host growth and enhancing soil health.

Functional differentiation is linked to the isolation site: rhizospheric isolates are specialised in the production of organic acids and siderophores, thereby facilitating the mobilization of phosphate and iron from the soil environment (Singh et al. 2022; Chen et al. 2024). Endophytic isolates focus on synthesising osmoprotectants and stress-related peptides that reinforce host plant tissues and improve tolerance to drought and salinity (Bouremani et al. 2024; Gu et al. 2024). These differentiated activities are crucial for supporting efficient nutrient cycling and enhancing plant resilience against environmental stress (Maciel-Rodríguez et al. 2025).

Initial consortium testing demonstrated significant metabolic synergy from the combination of *Bacillus pseudomycoides* (T4RCHM3) and *Klebsiella pneumoniae* (T6RCHM3). This consortium effectively enhanced the early growth of clove seedlings and phosphate-uptake efficiency (Shukla et al. 2022). This dual-strain formulation capitalises on the oxidative-stress tolerance characteristic of *Bacillus* and the high phosphate-solubilisation capacity of *Klebsiella*, positioning it as an optimal bioinoculant candidate for tropical marginal soils. These findings align with PGPR research on other tropical spice crops, where local microbial consortia have been reported to increase nutrient efficiency and tolerance to abiotic stress (Timofeeva et al. 2023).

Further proteomic validation substantiates the agronomic relevance of the observed PGPR traits by confirming the direct involvement of nitrogenase and phosphatase enzymes

in microbe-plant interaction (Flores et al. 2025). Specific isolates (e.g., T1RCHM27 and T6RCHM3) excel in organic-matter decomposition and nutrient mobilization (Singh et al. 2022), while another (T4RCHM3) demonstrates strong stress-mitigation potential through high catalase activity (Gu et al. 2024). Intra-strain functional diversity is influenced by host-tissue affinity, inter-microbial interactions, and specific metabolic repertoire. Strong host-microbe compatibility is central to enhancing plant performance and ecological adaptation (Rasul et al. 2024). A microbial consortium approach based on specific growth-promoting features is superior to one based purely on taxonomic identity. Therefore, the integration of physiological, enzymatic, and phylogenetic data provides a strong foundation for designing adaptive biofertilizers that sustainably support clove productivity and the resilience of tropical agroecosystems.

In conclusion, this study provides an integrative functional and molecular characterization of culturable endophytic and rhizospheric bacteria associated with *S. aromaticum* in Central Maluku, Indonesia, highlighting their value as PGPR candidates for tropical biofertilizer development. Eleven morphologically distinct isolates were recovered, with a clear predominance of rhizospheric strains (9 isolates) relative to leaf endophytes (2 isolates). Across physiological screening, three strains emerged as multifunctional. T5RCHM21 combined high IAA production and phosphate solubilization with positive nitrogen-fixation traits and tolerance to alkaline conditions (up to pH 10), T6RCHM3 exhibited outstanding phosphate solubilization (highest PSI) and strong cellulolytic activity, and T4RCHM3 demonstrated very strong catalase activity with measurable amylase and protease indices, indicating stress-mitigation capacity. 16S rRNA analysis dominated by *Bacillus*, with additional *Brevibacterium*, *Klebsiella*, while functional divergence among closely related taxa underscored that bioinoculant selection should prioritize phenotype-based performance rather than taxonomy alone. These findings support consortium-oriented strategy to assemble ecologically compatible strains that integrate nutrient mobilization and stress tolerance. Future work should focus on higher-resolution genomic identification, formulation development, and field validation to confirm efficacy under diverse tropical conditions.

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