

RESEARCH PAPER

Characterization of novel rhizosphere bacteria isolated from *Vanda tricolor* Lindl. var. *Suavis* Orchids in Mount Merapi Indonesia

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Academic editor: Syed Amir Ashraf ♦ Received 18 May 2025 ♦ Accepted 3 December 2025 ♦ Published 5 February 2026

Abstract

Vanda tricolor Lindl. var. *Suavis* is an orchid indigenous to Mount Merapi, Yogyakarta, Indonesia. Conservation efforts are necessary due to its diminishing presence in its natural habitat, which is caused by recurrent eruptions and exploitation by irresponsible parties. Ex situ conservation initiatives using in vitro culture propagation have been implemented; however, the growth of *Vanda tricolor* in acclimatization remains slow, as reflected by minimal increases in plant height, leaf length, leaf width, root length, and root diameter. Consequently, efforts to promote the development of *Vanda tricolor* must be implemented, especially using bacteria that are symbiotic with orchid roots. The obtained characterization data could assist in the future selection of bacteria that can promote the growth of *Vanda tricolor* orchids. This study aims to identify and characterize bacteria from the roots and rhizosphere of *Vanda tricolor* orchids. The results indicated that fourteen isolates were isolated, of which eight showed nitrogen fixation and nitrification capabilities. The isolates were identified as members of the genus *Bacillus* by 16S rRNA gene sequencing, including *Bacillus cereus*, *Bacillus toyonensis*, *Bacillus paramycoides* and *Bacillus* sp. Phylogenetic research revealed that these isolates grouped within the *Bacillus cereus* sensu lato complex, signifying significant genetic similarity and possible ecological functions in enhancing plant growth. The results indicate that *Bacillus* isolates from the *Vanda tricolor* rhizosphere have beneficial properties for bioinoculant production aimed at promoting orchid growth. Future research emphasizing physiological and genetic validation is essential to verify their working processes and enable safe application in orchid conservation efforts.

Keywords

Epiphyte, identification, microbe, nitrogen fixation, rhizosphere

Introduction

Orchidaceae is the largest and most widespread family within the Angiospermae subdivision, with two-thirds consisting of epiphytic orchids predominantly found in tropical forests. *Vanda tricolor* Lindl. var. *Suavis* is an orchid native to Mount Merapi, Yogyakarta, Indonesia; con-

servation efforts are essential due to its diminishing presence in its natural habitat, which is attributed to frequent eruptions and exploitation by irresponsible parties (Dwiyani 2013; Rineksane et al. 2020; Semiarti et al. 2022). Ex situ conservation initiatives using in vitro culture propagation have been implemented through shoot induction and multiplication, which employ organogenesis and embry-

ogenesis (Dwiyaning 2013; Rineksane et al. 2021; Rineksane et al. 2022; Ashihah et al. 2022). These efforts have resulted in acclimatized plantlets being successfully established in their native environment (Rineksane et al. 2023). The in vitro growth and post-acclimatization of *Vanda tricolor* remain slow, which requires around 2 years for the *Vanda tricolor* plantlets to acclimate (Rineksane et al. 2023). The growth of *Vanda tricolor* orchids in vitro culture is comparatively slow, as evidenced by minimal increases in plant height, leaf length, leaf width, root length, and root diameter during the in vitro incubation period (Dwiyaning 2013; Rineksane et al. 2021; Rineksane et al. 2022; Ashihah et al. 2022), as well as during the acclimatization phase (Rineksane et al. 2023). This slow growth delays the return of plantlets to their original habitat as a conservation measure for *Vanda tricolor* orchids. Hence, one strategy to enhance the growth of *Vanda tricolor* involves the use of symbiotic bacteria that promote plant development.

Plants serve as hosts to colonies of microbes, including bacteria. These bacteria contribute to plant growth and protect against infections (Cig and Yilmaz 2017; Bezerra et al. 2019; Pujasatria et al. 2020; Zhu et al. 2022; Quijia-Lamina et al. 2023). Consequently, the growth of *Vanda tricolor* can be enhanced by employing symbiotic bacteria in its roots. Symbiotic bacteria in the roots enhance orchid growth by performing nitrogen fixation (Andrade et al. 2023; Silveira et al. 2023) and producing beneficial hormones (Tsavkelova et al. 2007; Tsavkelova et al. 2016; Alibrandi et al. 2021; Shah et al. 2021; Yadav et al. 2022; Andrade et al. 2023; Fang et al. 2023) and enzymes (Andrade et al. 2023). This initiative represents an innovative approach to accelerate the in vitro growth of *Vanda tricolor* orchids as part of an ex-situ conservation effort. Symbiotic bacteria can be developed into a biological agent formulation that promotes plant development. The application of symbiotic bacteria to enhance orchid growth has been conducted on *Oncidium varicosum* (Bezerra et al. 2019), *Cattleya walkeri* (Andrade et al. 2023), *Eulophia alta* (Johnson et al. 2007), *Dendrobium catenatum* (Wu et al. 2012), *Dendrobium officinale* (Teixeira da Silva et al. 2015), and *Laelia* (Mayo-Mosqueda et al. 2022).

Bacteria that are symbiotic with roots and enhance orchid root growth have been isolated and identified (Tsavkelova et al. 2007; Pavlova et al. 2017; Gontijo et al. 2018; Huang et al. 2018; Shah et al. 2018; Jagannath et al. 2019; Altinkaynak and Ozkoc 2020; Herrera et al. 2020; Kaur and Sharma 2021; Suresh et al. 2023; Yadav et al. 2023). However, such isolation and identification have not been conducted on *Vanda tricolor* orchids. Therefore, this study aims to isolate and characterize bacteria in the roots and rhizosphere of *Vanda tricolor* orchids. The acquired characterization data may serve as the basis for the future selection of bacteria that can enhance the growth of *Vanda tricolor* orchids. The assessment of morphological traits continues to be employed in taxonomy, as noted by Buchanan and Gibbons (1974). Morphological criteria include the morphology of colonies in growth media and

cell morphology observable under a microscope at specific magnifications (Cappucino and Sherman 1987). The morphological properties of cell colonies in the growth medium encompass color, shape, size, and position. According to Campbell et al. (2002), Gram staining remains a crucial technique in bacterial taxonomy. It is used to categorize members of the bacterial domain into two groups based on their cell wall composition. Gram-positive bacteria have less complex cell walls with a significant quantity of peptidoglycan, while Gram-negative bacteria have cell walls with less peptidoglycan and greater structural complexity.

Beside morphological and physiological characterization, bacterial species were also identified molecularly by the 16S rRNA gene. 16S rRNA gene sequencing is a standard method for examining microbial diversity across many habitats, including plant tissues. This technique involves the amplification of the 16S rRNA gene with universal primers and sequencing the resultant amplicons to ascertain bacterial species (Yadav et al. 2018; Simmons et al. 2018).

Materials and methods

Study area

This study used the roots and rhizosphere of *Vanda tricolor* orchids, which were sourced from the slopes of Mount Merapi, specifically in the Turgo region, as illustrated in Fig. 1.

Material preparation

The materials utilized for bacterial isolation consisted of the roots and rhizosphere of *Vanda tricolor* orchids collected from the Merapi orchid conservation garden located on the slopes of Mount Merapi, specifically in the Turgo area, Pakem District, Sleman Regency, Special Region of Yogyakarta, Indonesia, as shown in Fig. 2. The A, B, C, and D codes for the samples indicate the various areas where the roots and rhizosphere of the *Vanda tricolor* orchids grow or adhere. *Vanda tricolor* is an epiphytic orchid that adheres to trees or decomposing wood rather than growing in soil. Code A signifies that *Vanda tricolor* adheres to fern roots, code B indicates a combination of charcoal and moss, code C refers to a decomposing log, and code D also refers to a mixture of charcoal and moss.

Isolation of bacteria from the roots of *Vanda tricolor* orchid

One gram of bacteria attached to the roots of *Vanda tricolor* orchid plants was suspended in 90 ml of sterile distilled water. In addition, 0.1 ml was inoculated onto the surface of Nutrient Agar (NA) media (Table 1), which comprised 3 g of beef extract, 5 g of peptone, and

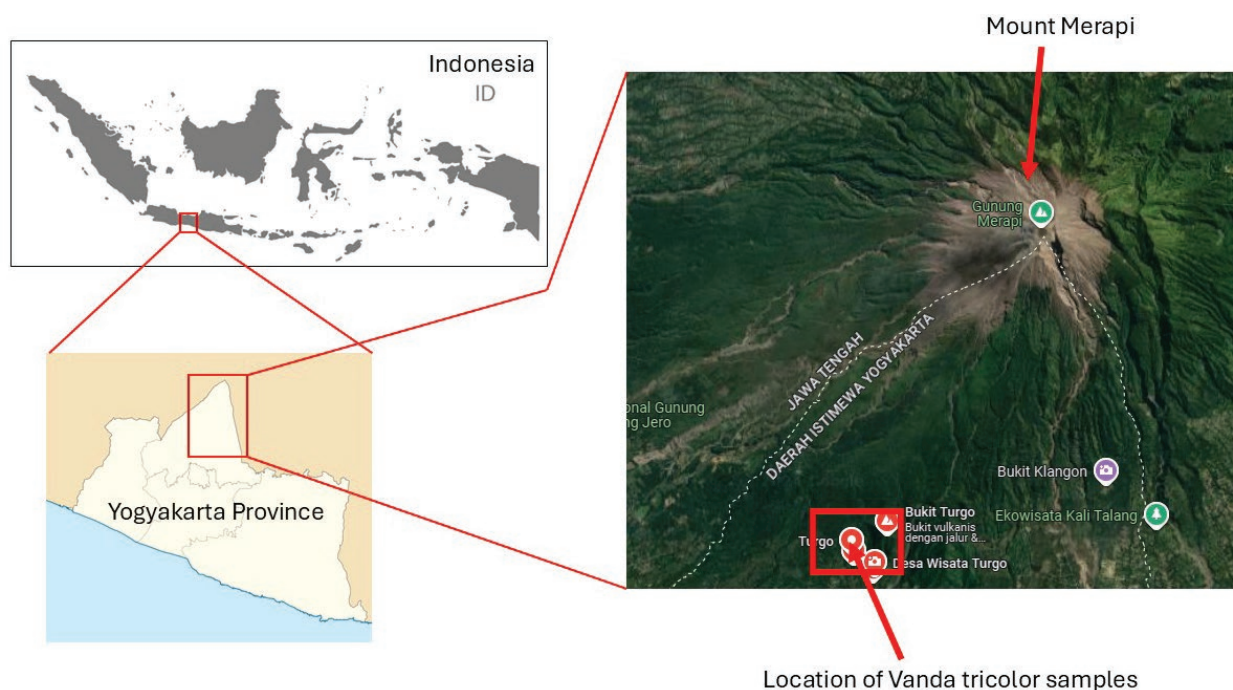


Figure 1. Location of root and rhizosphere sampling of *Vanda tricolor* orchid, in Turgu Area, Pakem District, Sleman Regency, Special Region of Yogyakarta, Indonesia (−7.586035098435313, 110.42076262916115).

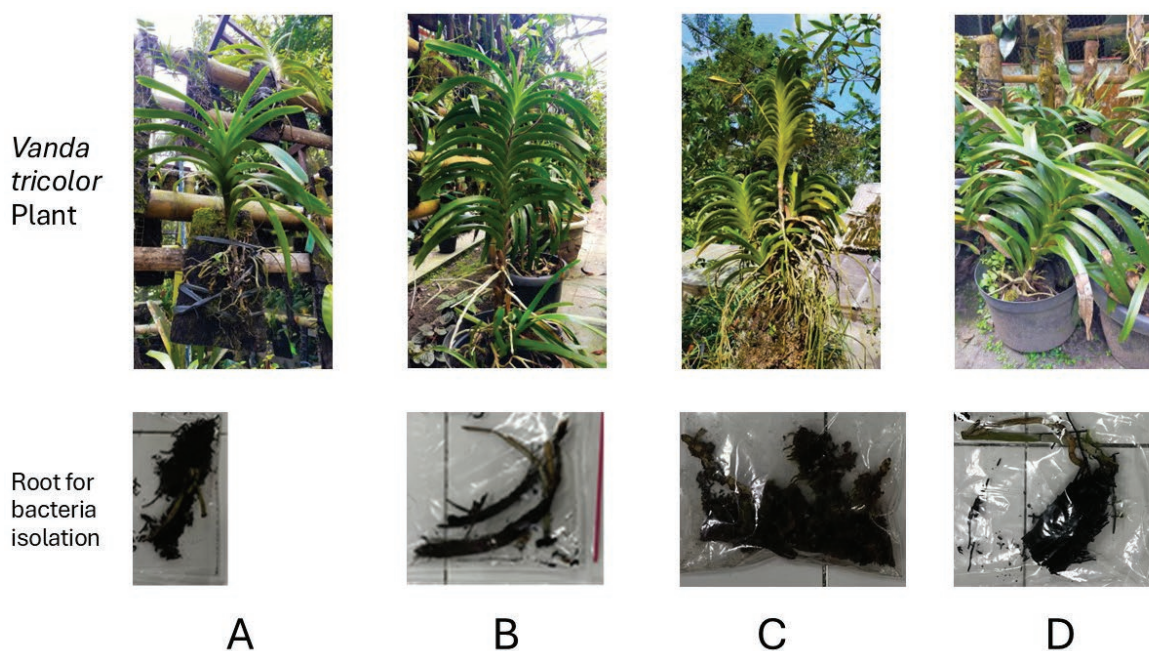


Figure 2. Four samples of *Vanda tricolor* orchid plants, along with their roots and rhizosphere, were used for bacterial isolation.

15 g of agar in 1,000 ml of distilled water. The culture was kept at room temperature for 48 h. Bacteria grown on NA media were subsequently purified, and the resulting pure isolate was transferred to NA media in a test tube. The bacterial isolate was further identified using colony morphology, cell characterization (gram staining, cell shape), physiological features (aerobics, catalase activity), and its potential for nitrogen fixation and nitrification capabilities.

Characterization of bacteria

Characterization of colony morphology

The morphological characterization of bacterial colonies was performed to investigate macroscopic characteristics. Bacterial suspensions were injected into NA media using streak plating with an ose needle and then incubated at 37 °C for 48 h. Isolated bacterial colonies were visually illustrated by shape, edge, color, elevation, and internal structure.

Gram staining and cell shape characterization

Gram staining was conducted to examine microscopic characteristics. Gram staining was performed on bacterial cultures incubated for 48 h and derived from pure bacterial isolates affixed to glass surfaces. The bacterial culture was treated with Crystal Violet stain for 1 min, followed by rinsing with running water. Subsequently, Lugol's iodine was applied for 1 min and subsequently rinsed again with running water. The bacterial culture was treated with 96% alcohol for 30 s, rinsed with running water, stained with Safranin for 2 min, and then washed again with running water. After the preparation dried, it was observed under a microscope. The classification of bacteria as Gram-positive or Gram-negative was determined through the staining of the specimen. Purple denotes gram-positive bacteria, while red signifies gram-negative bacteria. The morphology of bacterial cells can be classified as Cocci, Bacilli, or spirals.

Aerobics

Aerobics was observed in bacterial cultures incubated for 48 h and obtained from pure bacterial isolates cultivated in Liquid Nutrient (NC) media (Table 1). Aerobic bacteria are microorganisms that require oxygen to facilitate their growth. Their enzymatic mechanism needs oxygen as an electron acceptor during oxidative phosphorylation. Bacterial growth patterns based on oxygen requirements include: a). Obligate aerobes, b). Facultative Anaerobes, c). Obligate Anaerobes d). Aerotolerant Anaerobes, and e). Microaerophiles (Fig. 3).

Catalase test

The catalase test is valuable for identifying bacterial groups that produce the catalase enzyme. The catalase test involves transferring a loopful of bacterial isolate onto a drop plate containing a hydrogen peroxide (H_2O_2) reagent. The presence of air bubbles indicates a positive outcome, while their absence signifies a negative result.

Testing the Nitrogen-fixing ability of bacteria

Nitrogen-fixing bacteria were isolated using Jensen's medium (N-free) (Table 1). A total of 39.1 g of Jensen's medium was dissolved in 1,000 ml of distilled water, brought to a boil, and sterilized by autoclaving at 121 °C for 15 min. The purified bacterial isolates were introduced into Jensen's medium and cultured at ambient temperature for 48 h. The isolates that grew demonstrated the ability for nitrogen fixation.

Nitrification test

Nitrate and nitrite levels were measured in liquid nitrate media using the N-(1-Naphthyl Ethylene Diamine Dihydrochloride) technique. This measurement relies on the development of reddish hue resulting from a reaction between Sulfanilic Acid and N-(1-Naphthyl Ethylene Diamine Dihydrochloride).

Identification of bacteria

Bacterial isolates were characterized based on colony morphology, cell shape, Gram properties, physiology, and

Table 1. Composition of Nutrient Agar (NA), Liquid Nutrient (NC) and Jensen's Media.

Components	Nutrient Agar (NA) (%)	Liquid Nutrient (NC) (%)	Jensen's (g/L)
Pepton	0.5	0.5	–
Beef Extract	0.3	0.3	–
Agar	1.5	–	15.000
Sucrose	–	–	20.000
Dipotassium hydrogen phosphate (K_2HPO_4)	–	–	1.000
Manganese sulphate ($MnSO_4$)	–	–	0.500
Sodium chloride (NaCl)	–	–	0.500
Ferrous sulphate ($FeSO_4$)	–	–	0.100
Sodium molybdate (Na_2MoO_4)	–	–	0.005
Calcium carbonate ($CaCO_3$)	–	–	2.000

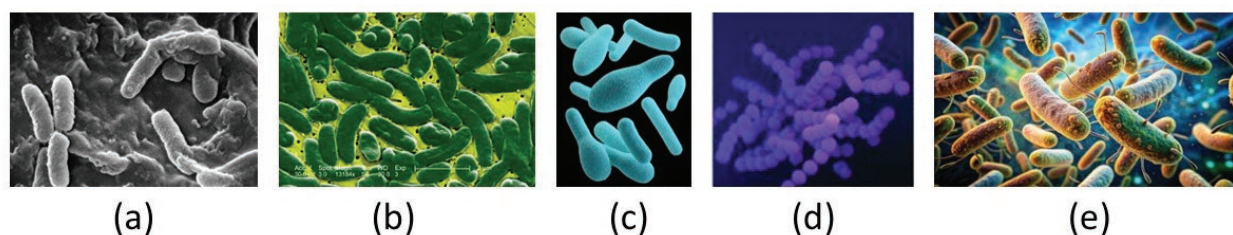


Figure 3. Bacterial growth patterns based on oxygen requirements: **a.** Obligate Aerobes (Carr, 2006); **b.** Facultative Anaerobes (Gettyimages.com, 2025); **c.** Obligate Anaerobes (Control Centers for Diseases, 2023); **d.** Aerotolerant Anaerobes (Biologyonline.com, 2025), and **e.** Microaerophiles (Stock.adobe.com, 2025).

their capacity for nitrogen fixation, and nitrification. The bacterial isolates were identified molecularly by the 16S rRNA gene. Bacterial DNA was extracted utilizing the DNA_Presto™ Mini gDNA Bacteria Kit. Additionally, Polymerase Chain Reaction (PCR) was conducted with the Gotax Master PCR mix (GoTaq master mix, 27_F, 1495_R, Template, NFW). Furthermore, Sanger Sequencing was conducted on the acquired samples utilizing the BigDye® Terminator v3.1 Cycle Sequencing Kit. Sequence data obtained was analyzed and edited manually to verify the sequence data validity. Homology analysis was performed by running BLAST tool provided by NCBI (National Centre of Biotechnology Information) at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Sequence analysis was also further continued with sequence alignment using GenomeNet.

Data resources

The data underpinning the analysis reported in this paper are deposited in the Dryad Data Repository at <https://doi.org/10.5061/dryad.cc2fqz6kz>.

Results and discussion

Colony morphological characteristics

Four samples of *Vanda tricolor* orchid roots and rhizosphere yield 14 bacterial isolates, which are designated as VTA1,

VTA2, VTA3, VTA4, VTA5, VTA6, VTA7, VTB1, VTB2, VTB3, VTC1, VTC2, VTD2, and VTD3. Among the 14 isolates, differentiation is based on colony color and form: 5 isolates are identified as milky white circular bacteria, 4 as yellow circular bacteria, 3 as cream curled bacteria, and 2 as cream amoeboid-irregular bacteria. Five identical isolates (VTA3, VTA5, VTB1, VTB2, and VTD3) are identified based on the milky white color and the shape of the circular colony (Table 2). Two isolates exhibit differences in their internal structure: isolate VTA3 is translucent, and isolate VTA5 is smooth. Meanwhile, the remaining isolates (VTB1, VTB2, and VTD3) show a coarsely granular internal structure. Two more isolates exhibit distinct edge shapes, namely, law convex (isolate VTD3) and elevation, specifically Ciliate (isolate VTB2), as detailed in Table 2.

According to the yellow color and the shape of the circular colony, four isolates are identified: VTA2, VTC1, VTC2, and VTD2 (Table 3). The isolates VTD2 and VTC1 exhibit differences in internal structure (transparent and coarsely granular) and coloration (greenish-yellow and yellow). In addition, VTD2 and VTA2 vary in edge shape (effuse and low convex) and color (greenish yellow and yellow). Furthermore, isolates VTA2 and VTC2 differ in colony form (circular and curled), elevation (entire and erose), and internal structure (transparent and coarsely granular), as illustrated in Table 3.

Three identical isolates (VTA1, VTA4, and VTB3) have a cream color and a curled colony form, and they differ only in edge shape (umbonate, raised, and convex). Meanwhile, VTA4 is additionally distinguished by its smooth interior structure, as presented in Table 4.

Table 2. Morphology of bacterial colonies obtained from the rhizosphere of *Vanda tricolor* based on milky white color and circular colony shape.

Isolate Code	Characteristics					
	Color	Diameter (mm)	Colony form	Edge shape	Elevation	Internal structure
VTA3	Milky white	01.01	Circular	Effuse	Entire	Translucent
VTA5	Milky white	01.07	Circular	Effuse	Entire	Smooth
VTB1	Milky white	01.06	Circular	Effuse	Entire	Coarsely granular
VTB2	Milky white	0.105	Circular	Effuse	Ciliate	Coarsely granular
VTD3	Milky white	02.01	Circular	Law convex	Entire	Coarsely granular

Table 3. Morphology of bacterial colonies obtained from the rhizosphere of *Vanda tricolor* based on yellow color and circular colony shape.

Isolate Code	Characteristics					
	Color	Diameter (mm)	Colony form	Edge shape	Elevation	Internal structure
VTD2	Greenish yellow	00.07	Circular	Effuse	Entire	Transparent
VTC1	Yellow	01.01	Circular	Effuse	Entire	Coarsely granular
VTA2	Yellow	01.01	Circular	Law convex	Entire	Transparent
VTC2	Yellow	01.01	Curled	Law convex	Erose	Coarsely granular

Table 4. Morphology of bacterial colonies isolated from the rhizosphere of *Vanda tricolor*: cream-colored with a curled colony shape.

Isolate Code	Characteristics					
	Color	Diameter (mm)	Colony form	Edge shape	Elevation	Internal structure
VTA1	Cream	01.05	Curled	Umbonate	Undulate	Coarsely granular
VTB3	Cream	01.01	Curled	Convex	Undulate	Coarsely granular
VTA4	Cream	01.06	Curled	Raised	Undulate	Smooth

The table illustrates that two isolates (VTA6 and VTA7) exhibited similar internal structures but varied in colony form (amoeboid and irregular), edge shape (convex rugose and convex papillate), and elevation (ciliate and undulate), as shown in Table 5.

The morphological characterization of bacterial colonies derived from the rhizosphere of *Vanda tricolor* roots (Tables 2–5) revealed four colony color variations: milky white, yellow, greenish-yellow, and cream, with milky white being the predominant color. Based on the colony morphology, four distinct forms were identified: round, curled, amoeboid, and irregular, with circular being the most prevalent. Seven varieties of edge shapes were identified based on the colony's edge: effuse, low convex, umbonate, convex, raised, convex rugose, and convex papillate, with effuse being the predominant shape. Four elevation variations were observed in the colony: entire, ciliate, erose, and undulate, with the entire elevation being the most common. In addition, four interior structure variations were noted: translucent, smooth, coarsely granular, and transparent. The morphological characterization of these bacterial colonies aligns with Bergey's Manual of Determinative Bacteriology (Holt et al. 2000). The form of bacterial colonies might vary based on the growth media, leading to a comparable appearance of bacterial growth at initial observation (Shchegolkova et al. 2024). Munar et al. (2023) presented findings on the isolation of phosphate-solubilizing bacteria from the rhizosphere of corn plants, exhibiting diverse colony morphologies including circular, irregular, filamentous, and rhizoid forms, with coloration ranging from transparent to creamy and broken white. Likewise, studies by Husna et al. (2025) regarding indigenous rhizobacteria extracted from the shallot rhizosphere and Sudewi et al. (2020b) concerning phosphate-solubilizing bacteria from the aromatic rice rhizosphere showed variations in color, shape, edge, and elevation.

Cell characterization

Table 6 summarizes the cell characterization of 14 bacterial isolates, highlighting cell shape and Gram staining. The 10 Gram-positive bacterial isolates are Bacilli (VTA3, VTB1, VTD3, VTC1, VTA2, VTC2, VTA1, VTB3, VTA4, and VTA6), the three Gram-negative bacterial isolates are Bacilli (VTA5, VTB2, VTA7), and the one Gram-negative bacterial isolate is Cocci (VTD2).

Among the 14 bacterial isolates obtained from the rhizosphere of *Vanda tricolor* roots, 13 exhibited a Bacilli shape, whereas one displayed a Cocci shape (Table 6). This finding aligns with the assertion of Taib et al. (2023) that Bacilli is the predominant bacterial type in nature. This aligns with the argument stated by Risanti et al. (2025), who studied *Bacillus* from the vegetable rhizosphere for its effects on plant growth. Among the 13 Bacilli isolates, 10 were Gram-positive and three were Gram-negative. Campbell et al. (2002) also McCue and Bishop (2021) stated that Gram staining categorizes bacteria into two groups

according to their cell wall composition. Gram-positive bacteria possess simpler cell walls characterized by a substantial quantity of peptidoglycan, whereas Gram-negative bacteria contain less peptidoglycan and exhibit greater structural complexity. Barazandeh (2008) indicated that Gram-positive bacteria consist of 90% peptidoglycan and feature distinct components in their cell walls, including teichoic acid and lipoteichoic acid. Meanwhile, Halimursyadah et al. (2022) stated that the bacterial response to the Gram stain reaction is significant as it identifies the group or kind of bacteria. For example, the *Bacillus* group is a form of rhizobacteria that is good for plants and has a positive gram reaction. Simultaneously, the VTD2 isolate is cocci and Gram-negative, as shown in Table 6.

Cell physiology

The physiological traits of 14 bacterial isolates, determined by their aerobic and catalase characteristics, are summarized in Table 7. The findings indicate that all isolates are aerobic and catalase-positive.

The bacterial isolates are catalase positive, as indicated in Table 7, indicating that they can convert hydrogen peroxide into water and oxygen due to the presence of the catalase enzyme. Almost all organisms that require oxygen for their metabolic processes contain this enzyme (Sudewi et al. 2020a). This finding is consistent with the bacterial cell physiology observed in this study, thereby revealing that all bacterial isolates tested exhibited aerobic characteristics, indicating their requirement for oxygen in metabolism. According to Murali and Patel (2017) and Sahu et al. (2019), hydrogen peroxide is a component produced by bacteria during respiration. However, hydrogen peroxide is toxic and can harm the bacterial metabolic system. To protect themselves from the harmful effects of hydrogen peroxide, bacteria must break it down into different chemicals. This procedure is facilitated by catalase enzymes, which enables bacteria to decompose hydrogen peroxide into water and oxygen, preventing toxicity and allowing the bacteria to survive.

Bacterial potential for N fixation and nitrification capability

The nitrogen fixation, and nitrification capabilities of 14 bacterial isolates were assessed, identifying eight isolates (VTA1, VTA2, VTA3, VTA7, VTB2, VTC1, VTC2, and VTD2) with these abilities, as shown in Table 8.

According to Table 8, among the 14 isolates, eight bacterial isolates possess the capability to fix nitrogen and perform nitrification. This means that these 8 bacteria have the potential to increase the growth of *Vanda tricolor*, due to their ability to fix nitrogen, which is the main element in plant growth. Nitrogen is a vital nutrient for plants, characterized by its tendency to be depleted from the soil due to several factors, including volatilization, nitrification,

Table 5. Morphology of bacterial colonies isolated from the rhizosphere of *Vanda tricolor*: cream-colored with the same internal structure but different colony shapes, edges, and elevations.

Isolate Code	Characteristics					
	Color	Diameter (mm)	Colony form	Edge shape	Elevation	Internal structure
VTA6	Cream	05.03	Amoeboid	Convex rugose	Ciliate	Coarsely granular
VTA7	Cream	01.09	Irregular	Convex papillate	Undulate	Coarsely granular

Table 6. Morphology of bacterial cells isolated from the rhizosphere of *Vanda tricolor*, categorized by cell shape and Gram staining.

Isolate Code	Characteristics	
	Cell shape	Gram stain
VTA3	Bacilli	Positive
VTB1	Bacilli	Positive
VTD3	Bacilli	Positive
VTC1	Bacilli	Positive
VTA2	Bacilli	Positive
VTC2	Bacilli	Positive
VTA1	Bacilli	Positive
VTB3	Bacilli	Positive
VTA4	Bacilli	Positive
VTA6	Bacilli	Positive
VTA5	Bacilli	Negative
VTB2	Bacilli	Negative
VTA7	Bacilli	Negative
VTD2	Cocci	Negative

Table 7. Physiology of bacterial cells isolated from the rhizosphere of *Vanda tricolor*, characterized by aerobics and catalase tests.

Isolate Code	Characteristics	
	Aerobics	Catalase
VTA3	Aerob	Positive
VTB1	Aerob	Positive
VTA5	Aerob	Positive
VTB2	Aerob	Positive
VTD3	Aerob	Positive
VTD2	Aerob	Positive
VTC1	Aerob	Positive
VTA2	Aerob	Positive
VTC2	Aerob	Positive
VTA1	Aerob	Positive
VTB3	Aerob	Positive
VTA4	Aerob	Positive
VTA6	Aerob	Positive
VTA7	Aerob	Positive

denitrification, leaching, and erosion (Sari and Prayudyaningsih 2015; Sandhu et al. 2021). The assessment of nitrogen-fixing bacteria was conducted using a selective medium devoid of nitrogen compounds, necessitating the bacteria to assimilate atmospheric nitrogen. Microbial activity is essential for sustaining the availability of macro-nutrients critical for plants, particularly nitrogen. While plants cannot directly utilize the substantial quantities of

Table 8. Results of bacterial potential assessment for nitrogen fixation and nitrification abilities.

Isolate Code	Characteristics	
	N Fixation Capability	Nitrification Capability
VTA3	Positive	Positive
VTB1	Negative	Negative
VTA5	Negative	Negative
VTB2	Positive	Positive
VTD3	Negative	Negative
VTD2	Positive	Positive
VTC1	Positive	Positive
VTA2	Positive	Positive
VTC2	Positive	Positive
VTA1	Positive	Positive
VTB3	Negative	Negative
VTA4	Negative	Negative
VTA6	Negative	Negative
VTA7	Positive	Positive

nitrogen in the environment, nitrifying bacteria transform nitrogen into ammonia, subsequently converting ammonia into nitrite and nitrite into nitrate (Kiding et al. 2015; Manthos et al. 2023). Nitrifying bacteria enhance organic matter and nutrient availability by supplying nitrate, which is readily absorbed by plant roots. Simarmata et al. (2023) stated that various types of bacteria could fix Nitrogen gas to produce the organic nitrogen that could support plants in terms of nitrogen requirement. The enzymes that are responsible for nitrogen fixation are nitrogenases.

Based on the results of isolation and characterization, 14 bacterial isolates were obtained from the rhizosphere of *Vanda tricolor* orchids. However, only eight bacterial isolates demonstrated the potential to enhance the growth of *Vanda tricolor*. This capability is attributed to their ability to fix atmospheric nitrogen and convert it into nitrates, which plants can absorb through nitrification processes. Table 9 illustrates a comprehensive evaluation of these eight isolates, detailing their colony morphology, cell shape, Gram characteristics, physiology, and nitrogen fixation, and nitrification abilities.

Table 9 indicates that the eight selected bacterial isolates exhibited different colors: yellow (VTA2, VTC1, and VTC2), cream (VTA1 and VTA7), milky white (VTA3 and VTB2), and greenish yellow (VTD2). The diameter of the colonies ranged from 0.7 mm to 1.9 mm. The predominant colony morphology was circular (VTA2, VTA3, VTB2, VTC1, VTD2), with other forms being curled (VTA1, VTC2) and irregular (VTA7). The colony edges predominantly exhibited an effuse shape (VTA3, VTB2,

Table 9. Results of the identification of colony morphology, cell shape, gram staining, physiological properties, and the capacity of bacteria for nitrogen fixation, and nitrification.

Isolate Code	Characteristics											
	Morphology						Cell		Physiology		Growth Inducer	
	Color	Diameter (mm)	Colony form	Edge shape	Elevation	Internal structure	Cell shape	Gram stain	Aerobics	Catalase	N fixation	Nitrification
VTA1	Cream	1.5	Curled	Undulate	Umbonate	Coarsely granular	Bacilli	(-)	Aerob	(+)	(+)	(+)
VTA2	Yellow	1.1	Circular	Entire	Law convex	Transparent	Bacilli	(+)	Aerob	(+)	(+)	(+)
VTA3	Milky white	1.1	Circular	Effuse	Entire	Translucent	Bacilli	(+)	Aerob	(+)	(+)	(+)
VTA7	Cream	1.9	Irregular	Conver papillate	Undulate	Coarsely granular	Bacilli	(-)	Aerob	(+)	(+)	(+)
VTB2	Milky white	1.5	Circular	Effuse	Ciliate	Coarsely granular	Bacilli	(-)	Aerob	(+)	(+)	(+)
VTC1	Yellow	1.1	Circular	Effuse	Entire	Coarsely granular	Bacilli	(+)	Fluctuative Aerob	(+)	(+)	(+)
VTC2	Yellow	1.1	Curled	Law convex	Erose	Coarsely granular	Bacilli	(+)	Aerob	(+)	(+)	(+)
VTD2	Greenish Yellow	0.7	Circular	Effuse	Entire	Transparent	Cocci	(-)	Aerob	(+)	(+)	(+)

VTC1, and VTD2), alongside undulate (VTA1), entire (VTA2), conver papillate (VTA7), and law convex (VTC2) forms. The colony elevation shows variability, including umbonate (VTA1), law convex (VTA2), entire (VTA3, VTC1, VTD2), undulate (VTA7), ciliate (VTB2), and erose (VTC2). The predominant internal structure of the colony is coarsely granular (VTA1, VTA7, VTB2, VTC1, and VTC2), with transparent structures (VTA2, VTD2) and translucent elements (VTA3). Table 9 presents the cell characterization results for the eight bacterial isolates, revealing that nearly all the bacterial cells are bacilli, except for one isolate, VTD2, which is cocci. Among the eight bacterial isolates, four are Gram-positive (VTA2, VTA3, VTC1, and VTC2) and four are Gram-negative (VTA1, VTA7, VTB2, and VTD2). From a physiological perspective, nearly all bacterial isolates are aerobic, with one fluctuating aerobic isolate (VTC1). Furthermore, all bacterial isolates are confirmed to be catalase-positive based on the catalase test. In terms of enhancing the growth of *Vanda tricolor* orchids, all eight bacterial isolates demonstrate the capability to fix nitrogen and perform nitrification.

Identification of bacterial isolates based on 16S rRNA gene sequences

Molecular identification via 16S rRNA gene sequencing indicated that all bacterial isolates from the rhizosphere of *Vanda tricolor* are classified within the genus *Bacillus* (Table 10).

The sequence identity percentage varied from 89.59% to 100%, revealing a close relationship between these isolates and species such as *Bacillus wiedmannii*, *B. cereus*, *B. toyonensis*, and *B. paramycoides*. These organisms are phylogenetically categorized within the *Bacillus cereus* group. Sequence identities exceeding 97% generally indicate species-level similarity, whereas lower percentages

imply genus-level identification (Stackebrandt & Goebel, 1994). Consequently, isolates VTA1, VTA2, VTB2, VTC1, VTC2, and VTD2 may be confidently classified into recognized species, whereas isolate VTA3 (89.59%) and VTA7 were identified only at the genus level. The prevalence of *Bacillus* spp. in the rhizosphere of *Vanda tricolor* aligns with other studies indicating that this genus is frequently located in the roots of orchids and other horticultural species, however additional functional validation is required.

Functional potential of identified *Bacillus* species

The VTA1 isolate revealed 97.63% similarity to *Bacillus wiedmannii*. Rashid et al. (2025) stated that *B. wiedmannii* may promote plant growth in stressful circumstances. This isolate showed potential as a plant growth-promoting rhizobacterium for *Vanda tricolor*. Nonetheless, because of the lack of obvious species identification and the absence of direct functional data on orchids, additional measures to quantify PGPR activity and efficacy on *Vanda tricolor* are required prior to extensive application.

The isolates VTA2, VTC1, and VTD2 showed a remarkable 16S rRNA similarity ($\geq 99\%$) to *Bacillus cereus*, signifying that all three are part of the *B. cereus* sensu lato complex. Several *B. cereus* strains have been identified as plant growth-promoting rhizobacteria (PGPR) due to their production of indole-3-acetic acid (IAA), siderophores, phosphate solubilization, and antagonistic effects against plant pathogens, thereby potentially promoting root development and plant resilience, including in horticultural species such as tomato and rice (Solano-Álvarez et al. 2021; Kulkova et al. 2023). Due to the genetic diversity of *B. cereus* and the presence of toxin genes (e.g., *nhe*, *hbl*, *cytK*, and *ces*) in certain strains that may induce toxic effects in humans and animals, additional identification

Table 10. Molecular identification of bacterial isolates from the rhizosphere of *Vanda tricolor* based on 16S rRNA gene sequence analysis.

Isolate code	Predicted Species	Identity value (%)	NCBI Accession number
VTA1	<i>Bacillus wiedmannii</i>	97.63	MT634452.1
VTA2	<i>Bacillus cereus</i>	99.72	OL587994.1
VTA3	<i>Bacillus</i> sp.	89.59	EU685818.1
VTA7	<i>Bacillus</i> sp.	99.72	ON247933.1
VTB2	<i>Bacillus toyonensis</i>	100	MN421112.1
VTC1	<i>Bacillus cereus</i>	99.51	KU922452.1
VTC2	<i>Bacillus paramycoides</i>	99.79	OR394251.1
VTD2	<i>Bacillus cereus</i>	99.86	KX430857.1

via whole-genome sequencing and toxin gene screening is essential prior to its use as a biological agent (Zhou et al. 2024; Kowalska et al. 2024). Consequently, although the identification results indicate promising PGPR potential, safety verification and quantitative biological assessments remain necessary to confirm the advantages and safety of *B. cereus* isolates on the growth of *Vanda tricolor*.

Isolates VTA3 and VTA7, classified as *Bacillus* sp., show potential as plant growth-promoting rhizobacteria (PGPR), considering that the *Bacillus* genus is recognized for its ability to synthesize indole-3-acetic acid (IAA), solubilize phosphate, produce siderophores, and suppress pathogens via bioactive lipopeptides (Tsoetsi et al. 2022). The taxonomic identity of isolate VTA3, with a low similarity of approximately 89.6%, requires further validation through genomic analysis, whereas VTA7, with a similarity of 99.7%, needs more testing on *Vanda tricolor* to evaluate its capacity to enhance plant growth. Due to the strain-specific functionality of PGPR in *Bacillus*, it is essential to conduct quantifiable phenotypic assessments, including IAA synthesis, phosphate solubilization, siderophore activity, and antagonism tests prior to practical application (Rigamonte et al. 2010; Sagar et al. 2024).

The VTB2 isolate showed significant similarity to *Bacillus toyonensis*, a species within the *Bacillus cereus* sensu lato complex, which has been reported as safe and potentially advantageous as a plant growth promoter (PGPR). For example, the AA1EC1 strain of *B. toyonensis* may create IAA, siderophores, and solubilize phosphate, which enhances plant length, root weight, and aerial growth in plants such as tomato and carrot (Roca et al. 2024). Due to these characteristics, the VTB2 isolate indicates significant potential for enhancing the growth and survival of *Vanda tricolor*. However, additional verification procedures—such as phenotypic PGPR assays (hormone synthesis, phosphate solubilization, siderophore generation) and genomic safety confirmation—are required prior to practical use.

The VTC2 isolate has significant similarity to *Bacillus paramycoides*, a constituent of the *Bacillus cereus* sensu lato complex, which has been reported for its potential as a plant growth-promoting rhizobacteria (PGPR). Research indicates that *B. paramycoides* may synthesize siderophores, hydrolytic enzymes, and antagonistic chemicals targeting plant diseases, and possesses genes associated with phosphate solubilization and growth hormone pro-

duction (Mghazli et al. 2022). The VTC2 isolate showed the potential to enhance the growth and resistance of *Vanda tricolor*; nevertheless, phenotypic research is required to validate its efficacy.

The identification results reveal that bacterial isolates from the rhizosphere of *Vanda tricolor* are classified within the genus *Bacillus*, namely *B. wiedmannii*, *B. cereus*, *B. toyonensis*, and *B. paramycoides*, which are extensively reported for their potential as plant growth-promoting rhizobacteria (PGPR). These species are known for their ability to create indole-3-acetic acid (IAA), solubilize phosphate, synthesis siderophores, and generate antifungal lipopeptides that enhance root growth and plant resilience. However, the functional impact of these isolates on the growth of *Vanda tricolor* has not been empirically validated. Consequently, suggested subsequent actions include phenotypic evaluation of PGPR characteristics, inoculation trials on *Vanda tricolor* plantlets to evaluate growth impacts, genomic assessment to verify strain safety, particularly within the *B. cereus* sensu lato group, and bioinoculant formulations tested at greenhouse or field scales to ascertain their efficacy in enhancing plant growth and resistance.

Numerous studies have shown that bacterial populations in the rhizosphere of epiphytic orchids adapt to the unique climatic conditions of the volcanic region, including those on the slopes of Mount Merapi. This adaptability is essential for the survival and growth of orchids. Rhizosphere bacteria contribute to nutrient cycling, including nitrogen fixation, phosphate solubilization, and siderophore synthesis. Due of changes in the seasons and limited nutrition supply, these bacteria can also withstand abiotic stress (Du et al. 2025; Aini and Hanudin 2025).

Phylogenetic relationship of bacterial isolates obtained from the rhizosphere of *Vanda tricolor*

Phylogenetic study of 16S rRNA from seven bacterial isolates in the rhizosphere of *Vanda tricolor* revealed that all isolates were closely affiliated within the *Bacillus cereus* sensu lato complex, including *B. cereus*, *B. paramycoides*, *B. toyonensis*, and various *Bacillus* species (Fig. 4). The low genetic distance results suggested significant taxonomic similarity, indicating that the isolates were likely strains

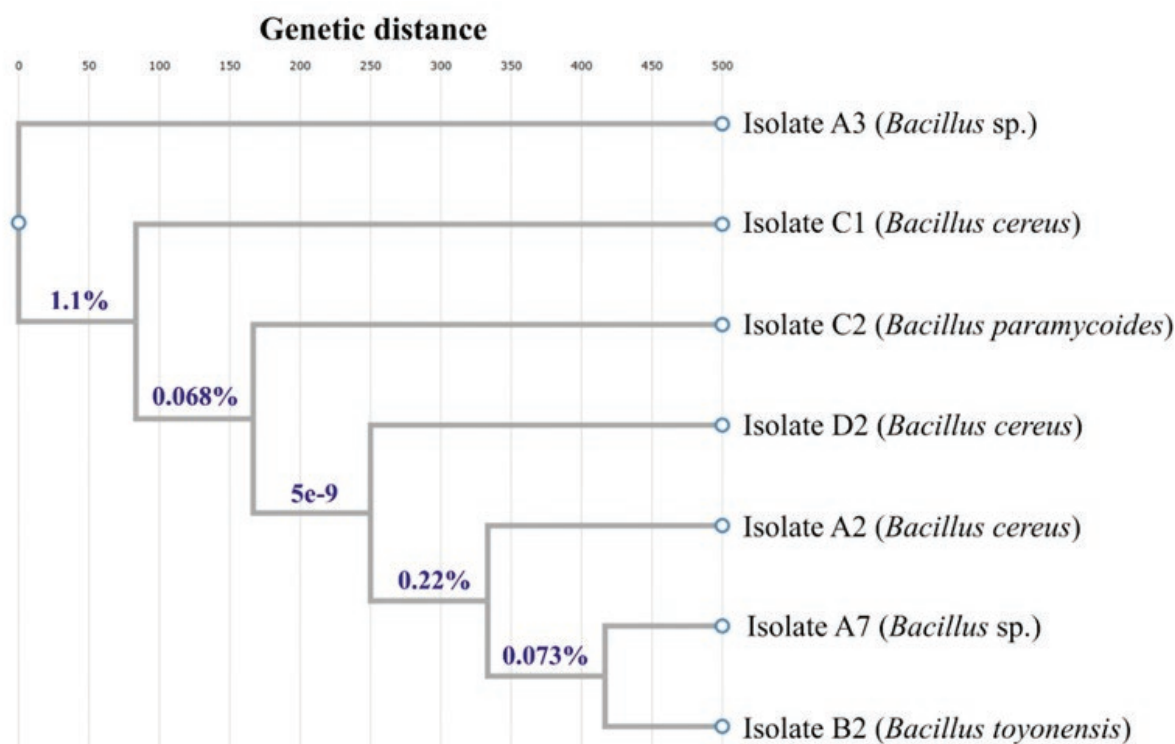


Figure 4. Phylogenetic relationship among seven *Bacillus* isolates from the rhizosphere of *Vanda tricolor* based on 16S rRNA gene sequences.

within the same species group. These results align with research conducted by Altinkaynak and Ozkoc (2020), which indicated the prevalence of the *Bacillus* genus in the rhizosphere and endophytes of diverse orchid species.

Species within the *Bacillus cereus* group are recognized as plant growth-promoting rhizobacteria (PGPR) capable of synthesizing IAA, solubilizing phosphate, producing siderophores, and generating antimicrobial compounds (Ait et al. 2013; Linnet 2024). Therefore, the discovered isolates possess the ability to enhance the development and resistance of *Vanda tricolor*. The subsequent recommended procedures involve doing biochemical and in planta assessments to evaluate the functional activity of PGPR, alongside genomic analysis to verify the safety and efficacy of the isolates prior to their application as bioinoculants in the conservation and culture of *Vanda tricolor*.

Conclusions

This research identified eight bacterial isolates from the rhizosphere of *Vanda tricolor*, primarily classified under the genus *Bacillus*, including *Bacillus cereus*, *Bacillus paramycoides*, *Bacillus toyonensis* and *Bacillus* sp. Phylogenetic research utilizing 16S rRNA sequences revealed significant genetic similarities, indicating close evolutionary links and potential functional synergy in enhancing plant development. These isolates are expected to promote nutrient mobilization and root development, indicating their potential function as plant growth-promoting rhizobacteria. Additional research is required to assess their direct

impact on *Vanda tricolor* growth via in vitro and greenhouse experiments to formulate effective bioinoculants that encourage orchid growth and conservation.

Authors' contributions

Innaka Ageng Rineksane designed the research, conducted experiments, and wrote the manuscripts; Agung Astuti assisted in the selection and characterization of symbiotic bacteria; Gatot Supangkat Samidjo helped with microbial growth; Pangesti Nugraheni proofread the manuscript; and Sumarsih assisted with the experiments. All authors played a role in the completion of the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

Acknowledgements

The authors express gratitude to the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia for providing research funding under the Regular Fundamental Research scheme (Contract Number 107/ES/PG.02.00/PL/2024). Appreciation is also extended to Mr. Musimin for supplying samples from the *Vanda tricolor* orchid and Dr. Siti Nur Aisyah, S.P. for helping in sequencing analysis.

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