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Isolation and Identification of *Bacillus stercoris* CMB2 with Nitrogen-Fixing and Phosphate Solubilizing Capabilities from Baby Maize Roots

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Abstract

Baby maize (*Zea mays* L.) is one of the important food crops in Asian regions and exhibits strong adaptability to adverse conditions across diverse agroecological zones. However, maize is commonly cultivated on nutrient-poor soils and under dry conditions, resulting in low fertilizer use efficiency and unstable baby maize yields across cropping seasons. Excessive application of chemical fertilizers, particularly nitrogen and phosphorus fertilizers, not only increases production costs but also contributes to soil degradation and environmental pollution. Beneficial endophytic bacteria, especially nitrogen-fixing and phosphate-solubilizing bacteria, have been recognized as sustainable biological solutions to enhance nutrient acquisition and improve crop productivity. The main objective of this study was to isolate, identify, and biologically characterize an endophytic bacterial strain possessing both nitrogen-fixing and phosphate-solubilizing capabilities from baby maize root tissues, with the aim of promoting plant growth and increasing baby maize yield under nutrient-deficient conditions. The study was conducted at the Central Laboratory, An Giang University, Vietnam. Among ten bacterial isolates screened, *Bacillus stercoris* CMB2 (*B. stercoris* CMB2) was successfully isolated, identified, and selected due to its outstanding abilities in atmospheric nitrogen fixation and solubilization of insoluble phosphorus compounds. The application of *B. stercoris* CMB2 demonstrated considerable potential for sustainable baby maize cultivation by improving soil nutrient status, enhancing fertilizer use efficiency, and increasing baby maize productivity in nutrient-poor soils.

Keywords: baby maize; *Bacillus stercoris*; nitrogen fixation; phosphate solubilization

1. Introduction

Baby maize (*Zea mays* L.), harvested at the immature stage prior to pollination and fertilization, has emerged as a high-value horticultural commodity in both domestic and international markets. Unlike grain maize, baby maize is cultivated for its tender ears, which are widely consumed as fresh vegetables or processed products [1]. Globally, baby maize production has expanded rapidly in Asia, Africa, and Latin America due to its short growth cycle (60–70 days), high market demand, and suitability for crop intensification systems. Countries such as India, Thailand, and China are leading producers and exporters, supplying fresh and canned baby corn to Europe and North America [2]. In Vietnam, baby maize cultivation has developed strongly in recent years, particularly in the Red River Delta and Mekong Delta regions, where it contributes significantly to farmer income diversification and rural economic development. Its adaptability to different agroecological zones and compatibility with crop rotation systems make it an important component of sustainable vegetable production chains [3,4].

Despite its economic importance, baby maize productivity has shown instability in recent years due to increasing environmental and agronomic constraints. Intensive cultivation practices aimed at maximizing short-term yield have led to excessive application of chemical fertilizers and pesticides

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[5]. Nitrogen (N) fertilizers, especially urea, are often applied at rates exceeding crop requirements, resulting in nutrient imbalance, soil acidification, nitrate leaching, and greenhouse gas emissions. Similarly, high phosphorus (P) inputs can lead to nutrient fixation in soil, reducing fertilizer-use efficiency and increasing production costs [6,7]. Recent reports indicate that prolonged chemical fertilization disrupts soil microbial diversity, diminishes beneficial rhizosphere populations, and accelerates soil degradation processes [8,9]. In Vietnam, the trend toward crop intensification and multiple annual cropping cycles further exacerbates soil exhaustion. Continuous monocropping without adequate organic amendments reduces soil organic matter, impairs soil structure, and lowers microbial biomass. Additionally, climate variability particularly prolonged droughts and irregular rainfall patterns have intensified abiotic stress in maize-growing regions [10,11]. Water deficit conditions impair nutrient uptake, reduce photosynthetic efficiency, and ultimately decrease yield and cob quality [12]. Combined with declining soil fertility, these stress factors threaten the sustainability of baby maize production systems. Therefore, strategies that enhance nutrient-use efficiency while restoring soil biological health are urgently required [13].

Sustainable agricultural development increasingly emphasizes environmentally friendly approaches that reduce dependency on synthetic agrochemicals. Biological soil improvement through the application of plant growth-promoting microorganisms is gaining recognition as a viable alternative [14,15]. Beneficial rhizosphere and endophytic bacteria can enhance plant growth through multiple mechanisms, including biological nitrogen fixation, phosphate solubilization, phytohormone production, and stress mitigation. Among these traits, nitrogen fixation and phosphate solubilization are particularly critical for maize cultivation, given the high demand of this crop for N and P nutrients [16,17]. Nitrogen is a key macronutrient required for chlorophyll synthesis, protein formation, and overall plant development. Biological nitrogen fixation, mediated by diazotrophic bacteria possessing nitrogenase enzymes, converts atmospheric N_2 into bioavailable ammonium forms [18]. This process can partially substitute for synthetic N fertilizers, thereby reducing environmental pollution and production costs. Recent studies have demonstrated that endophytic nitrogen-fixing bacteria associated with maize roots can significantly improve plant biomass and nutrient uptake under reduced fertilizer regimes [19,20]. Phosphorus, although abundant in many soils, often exists in insoluble forms unavailable to plants. Phosphate-solubilizing bacteria enhances P availability by secreting organic acids and phosphatases that mobilize inorganic and organic phosphate compounds [16,21]. This microbial-mediated transformation increases P-use efficiency and promotes root development. Integrated N-fixing and P-solubilizing bacteria have shown synergistic effects in improving crop productivity while lowering chemical fertilizer inputs [22,23].

Among beneficial bacterial genera, *Bacillus* species have attracted considerable attention due to their resilience, spore-forming ability, and multifunctional plant growth-promoting traits [24]. Members of the genus *Bacillus* are well known for their capacity to colonize plant roots, tolerate environmental stress, and produce bioactive metabolites. Several Bacillus strains have been reported to possess both nitrogen-fixing and phosphate-solubilizing capabilities, making them promising candidates for biofertilizer development [18,24]. Furthermore, endophytic *Bacillus* strains can establish stable associations within plant tissues, offering enhanced protection and nutrient acquisition efficiency compared with free-living rhizobacteria [25]. However, the effectiveness of microbial inoculants is often strain-specific and influenced by environmental conditions. Indigenous microorganisms isolated from local agroecosystems are generally better adapted to regional soils and climatic factors [26]. Therefore, isolating and characterizing native endophytic bacteria from baby maize roots represents a strategic approach to identify efficient plant growth-promoting strains. Molecular identification, particularly through 16S rRNA gene sequencing, provides reliable taxonomic resolution and supports the selection of functional strains for further agronomic evaluation [27].

In this context, the present study focuses on the isolation and characterization of the endophytic bacterium *B. stercoris* CMB2 exhibiting nitrogen-fixing and phosphate-solubilizing traits. The

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rationale is that a locally adapted multifunctional strain may enhance nutrient availability, improve plant growth, and contribute to sustainable baby maize production under reduced chemical input conditions. By integrating microbiological screening, molecular identification, and functional evaluation, this research aims to provide a scientific basis for developing environmentally friendly biofertilizer formulations tailored to Vietnamese baby maize systems. Overall, transitioning from chemically intensive practices to biologically enhanced production models is essential for maintaining soil health, ensuring long-term productivity, and mitigating environmental risks. The exploitation of endophytic bacteria with dual nitrogen-fixing and phosphate-solubilizing capacities represents a promising pathway toward sustainable baby maize cultivation in Vietnam and beyond.

2. Materials and Methods

2.1. Collection and Processing of Baby Maize Root Samples

Baby maize root samples were collected from Cho Moi commune, An Giang province, Vietnam. Immediately after sampling, roots were aseptically cut into small segments using sterile scissors without prior washing to preserve the native rhizosphere soil layer. The root fragments were then macerated and homogenized in approximately 100 mL of sterile distilled water to release both epiphytic and endophytic bacterial populations. The resulting suspension was transferred into centrifuge tubes and agitated on an orbital shaker for 30 min to ensure adequate cell dispersion. Subsequently, the mixture was centrifuged at 1,500 rpm for 1 min, and the supernatant containing bacterial cells was collected and spread onto yeast extract mannitol agar (YMA) medium [27,28]. Serial tenfold dilutions ranging from 10^{-2} to 10^{-6} were prepared, and aliquots from each dilution were plated in triplicate onto YMA plates. The inoculated plates were incubated at room temperature for 4–5 days. Colony formation was monitored and recorded, and distinct colonies were repeatedly subcultured to obtain pure bacterial isolates [24,30].

2.2. Isolation and Functional Assessment of B. stercoris CMB2

Nitrogen-fixing bacteria were isolated from rhizosphere soil and root tissues using serial dilution and selective culturing techniques. For rhizosphere soil, 10 g of fresh soil was suspended in 90 mL of sterile distilled water and homogenized by shaking at 150 rpm for 20–30 min. The suspension was serially diluted up to 10^{-6}. Aliquots (100 µL) of appropriate dilutions were spread onto nitrogen-free selective medium (N-free agar or Jensen's medium) to screen for diazotrophic bacteria. Plates were incubated at 30 °C for 3–5 days under aerobic conditions. Distinct colonies showing typical morphological characteristics of free-living nitrogen fixers were selected and repeatedly streaked on fresh medium to obtain pure cultures [24].

For endophytic bacterial isolation, baby maize roots were first washed thoroughly under running tap water to remove adhering soil particles. Surface sterilization was performed by sequential immersion in 70% ethanol for 1 min, followed by 2% sodium hypochlorite solution for 3–5 min, and rinsed five times with sterile distilled water. The effectiveness of surface sterilization was verified by imprinting sterilized roots onto nutrient agar plates to confirm the absence of microbial growth [28,31].

Sterilized root tissues (approximately 1–2 g) were aseptically macerated in sterile phosphate-buffered saline using a mortar and pestle. The homogenate was serially diluted, and aliquots were spread onto nitrogen-free agar medium. Plates were incubated at 30 °C for 3–5 days. Emerging colonies were selected based on distinct morphology and purified through repeated streaking [20,28].

Pure isolates were maintained on yeast extract mannitol agar (YMA) slants at 4 °C for short-term storage and preserved in 20% glycerol at −80 °C for long-term storage. Preliminary screening for nitrogen-fixing ability was conducted by observing bacterial growth on nitrogen-free medium and confirming ammonia production and/or acetylene reduction activity in subsequent assays [18,28].

Rhizosphere Bacterial Isolation Procedure: Approximately 1–2 g of fresh baby maize root samples was weighed for bacterial isolation. As root tissues may harbor epiphytic contaminants, surface

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sterilization was performed prior to isolation. Roots were carefully washed under sterile water to remove adhering soil particles, avoiding mechanical damage. The samples were then immersed in 95% ethanol for 3 min, followed by surface sterilization in 0.1% $HgCl_2$ solution for 5 min. Subsequently, roots were rinsed five to six times with sterile distilled water to remove residual disinfectants. Sterilized root tissues were aseptically macerated using a sterile mortar and pestle, and 2 mL of sterile distilled water was added to obtain a homogenate [20,28,32]. One milliliter of the suspension was transferred into a test tube containing 9 mL of sterile distilled water to obtain a 10^{-1} dilution. Serial dilutions were prepared up to 10^{-4} and 10^{-5}. From each dilution, 100 µL aliquots were spread onto yeast extract mannitol agar (YMA) plates previously sterilized at 121 °C for 15 min. Each dilution was plated in triplicate. The inoculated plates were incubated at 28–30 °C for 3–7 days to allow the development of discrete colonies [28,33].

Purification of Isolates: Selected colonies were repeatedly streaked onto fresh solid medium to obtain single colonies. The purification process was performed two to three times to ensure culture homogeneity. Pure isolates were subsequently transferred onto agar slants for preservation and further characterization [28,34].

2.3. Morphological Characterization of B. stercoris CMB2

Colony morphology was examined visually after incubation on YMA medium. Characteristics including color, shape, elevation, margin type, surface texture, and colony size were recorded.

Wet Mount Preparation: A wet mount slide was prepared following the drop-slide method described by Oyeleke and Manga (2008). A small amount of fresh bacterial culture was suspended in a drop of sterile distilled water on a clean glass slide and covered with a coverslip. Cell morphology and motility were observed under a light microscope at 400× magnification [35].

Measurement of Cell Size: After determining cell shape and motility, bacterial cell dimensions were measured using an ocular micrometer calibrated with a stage micrometer under a light microscope, following the guidelines of Osiro et al., [36].

Gram Staining: Nitrogen-fixing bacterial isolates were cultured on YMA medium, and colonies appearing after 2–7 days were used for staining. A small amount of bacterial biomass was transferred with a sterile inoculating loop into a drop of sterile distilled water on a glass slide to prepare a smear. The smear was heat-fixed over an alcohol flame. Crystal violet was applied to the fixed smear for 30 s, followed by gentle rinsing with distilled water and air drying. Lugol's iodine solution was then added for 1 min, rinsed with water, and allowed to dry. Decolorization was performed using ethanol for approximately 30 s until the stain was removed. The slide was subsequently rinsed with distilled water and air-dried. Counterstaining was performed with safranin for 1 min, followed by gentle rinsing with water and air-drying. The stained smears were examined under a light microscope to observe cellular morphology. Gram-negative bacteria appeared pink due to safranin uptake, whereas Gram-positive bacteria retained the crystal violet–iodine complex and appeared purple [37].

2.4. Biochemical Characterization

Motility Test: Bacterial motility was determined using the semi-solid agar stab method. Motile bacteria are characterized by diffuse growth extending outward from the stab line, whereas non-motile bacteria grow only along the line of inoculation. A sterile inoculating needle was used to stab-inoculate the bacterial culture into semi-solid YMA medium. The tubes were kept in an upright position and incubated at 30 °C. Motility was assessed after 3 days based on the pattern of bacterial growth [38].

Catalase Test: The catalase test is based on the ability of bacterial isolates to produce the enzyme catalase, which decomposes hydrogen peroxide (H_2O_2) into water and oxygen.

A small portion of a fresh bacterial colony was transferred onto the center of a clean glass slide using a sterile loop. A few drops of 3% H_2O_2 were added to cover the smear. Results were recorded after approximately 15 s. The formation of visible oxygen bubbles indicated a positive catalase reaction [39].

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2.5. Determination of Nitrogen-Fixing Ability

The nitrogen-fixing potential of rhizosphere isolates was assessed using complementary qualitative and quantitative methods. Qualitative screening was performed by culturing the isolates on nitrogen-free media, including Ashby's mannitol agar, NFb medium, and Burk's N-free medium. Growth under nitrogen-deficient conditions indicated putative diazotrophic capability [28].

Quantitative evaluation was conducted using the acetylene reduction assay (ARA), which measures the conversion of acetylene (C_2H_2) to ethylene (C_2H_4) by the nitrogenase enzyme complex (Hardy et al., 1968). Ethylene production was quantified by gas chromatography as an indirect indicator of nitrogenase activity. Total nitrogen accumulation was further determined using Kjeldahl digestion or CHN elemental analysis to estimate biologically fixed nitrogen [40].

Ammonia production was qualitatively examined by incubating cultures in peptone water at 30 °C for 60–80 h, followed by Nessler's reagent detection. For nitrogenase analysis, cells were pre-grown in YMA broth, transferred to nitrogen-free medium (OD_{600} = 0.8), and incubated at 30 °C with shaking (160 rpm), including an uninoculated control. Total nitrogen content was measured following centrifugation as described by Kifle et al. [41]. Environmental factors such as pH, temperature, oxygen availability, and carbon source were considered due to their influence on nitrogenase activity.

2.6. Evaluation of Phosphate-Solubilizing Capacities

The phosphate-solubilizing capacity of the bacterial isolates was evaluated following the protocol described by Nautiyal [42]. The National Botanical Research Institute's phosphate (NBRIP) medium developed by Nautiyal contains glucose and 5.0 g L^{-1} tricalcium phosphate (TCP) as the insoluble phosphorus source, along with magnesium chloride hexahydrate, magnesium sulfate heptahydrate, potassium chloride, ammonium sulfate, and distilled water. This medium is widely applied for assessing phosphate solubilization efficiency [43].

Phosphate solubilization on solid medium was determined by measuring the diameter of the clear halo formed around colonies after inoculation of a fresh suspension of B. stercoris CMB2 onto NBRIP agar plates. The solubilization index (SI) was calculated after 7, 14, and 21 days of incubation at 28 ± 2 °C using the formula [43,44]:

$$SI = \frac{CD + HD}{CD}$$

where CD represents the colony diameter and HD denotes the halo zone diameter. Quantitative estimation of soluble phosphate was conducted using a colorimetric assay in liquid NBRIP medium. Fifty milliliters of NBRIP broth supplemented with 0.5% TCP was inoculated with 200 µL of a freshly prepared bacterial suspension adjusted to an optical density (OD_{600}) of 0.8 (approximately 5 × 10^8 CFU mL^{-1}). Cultures were incubated at 28 ± 2 °C for 7 days under shaking conditions (180 rpm) [43–45]. Following incubation, cultures were centrifuged at 10,000 rpm for 10 min, and the supernatant was collected for soluble phosphorus quantification using the vanado molybdate yellow colorimetric method at 430 nm [45]. Measurements were recorded at 0, 3, and 7 days of incubation. All treatments were performed in triplicate.

2.7. Molecular Identification and Genetic Analysis

A single purified colony of *B. stercoris* CMB2 was transferred into sterile microcentrifuge tubes for total genomic DNA extraction using the GeneJET Genomic DNA Purification Kit (Thermo Scientific™), following the manufacturer's instructions. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) (Figure 1c), yielding an amplicon of approximately 1,334 bp. The obtained sequences were subsequently edited and aligned using MEGA software and compared with reference sequences available in the GenBank database through the BLAST algorithm. The nucleotide sequence of strain CMB2 was deposited in GenBank under accession number PX795046.1 and is publicly accessible

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(https://www.ncbi.nlm.nih.gov/nuccore/PX795046.1?report=gbwithparts&log$=seqview).
Phylogenetic tree (Figure 2) analysis revealed that *B. stercoris* CMB2 clustered within the genus *Bacillus*, thereby confirming its taxonomic affiliation. Moreover, *B. stercoris* CMB2 exhibited high sequence similarity to *stercorid* species and showed a close phylogenetic relationship with *Bacillus stercorid* SG25 [9,18,27].

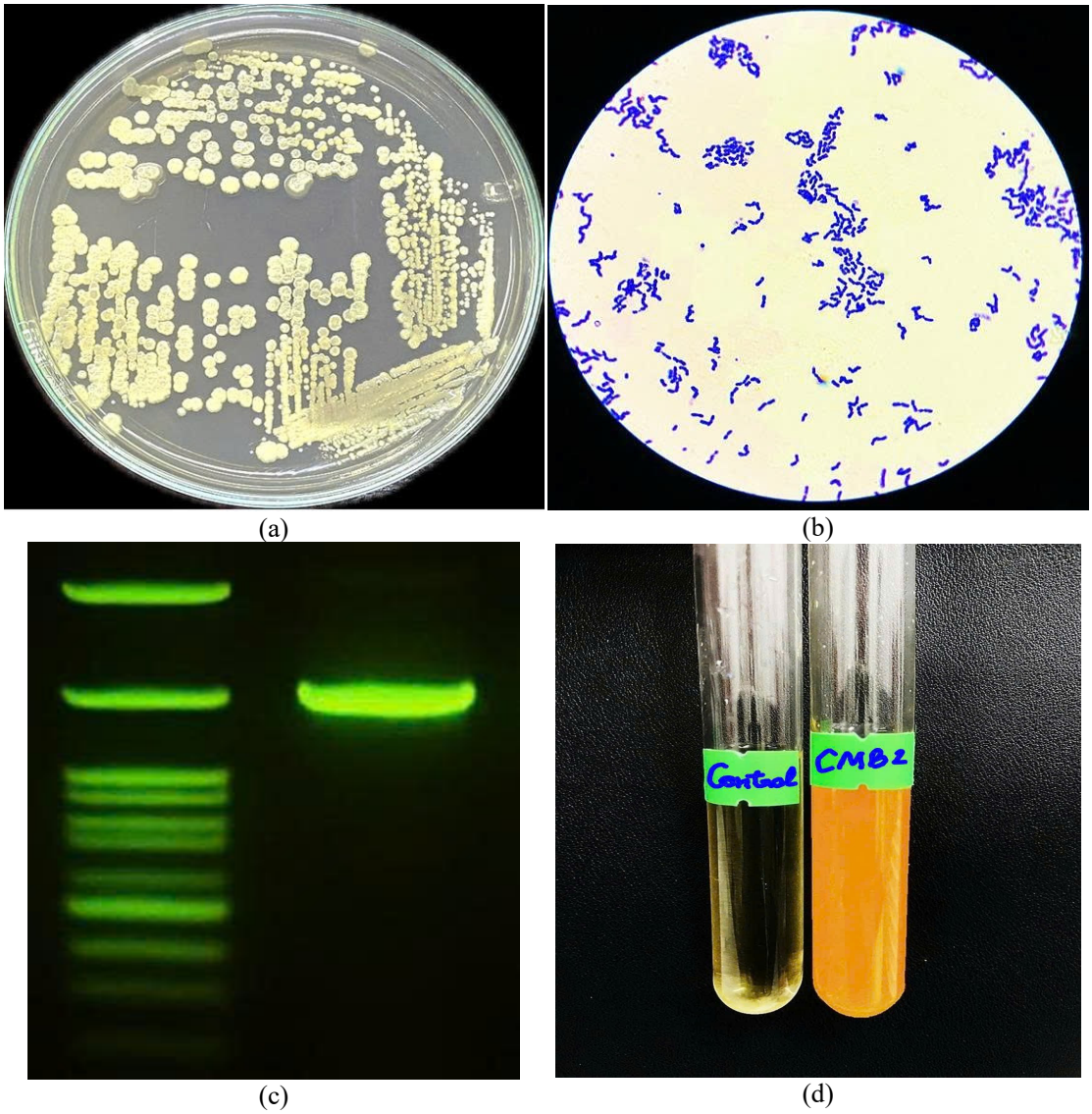

Figure 1. Morphological features and functional characterization of B. stercoris CMB2: (a) colony appearance on YMA medium; (b) cell morphology under light microscopy (100×); (c) agarose gel electrophoresis of the PCR-amplified 16S rRNA gene fragment visualized with a gel documentation system; and (d) qualitative assessment of ammonia production in peptone-enriched broth.

2.8. Phenotypic Characterization of B. stercoris CMB2

Strain CMB2 was subjected to morphological and biochemical characterization following purification. Gram staining, catalase, and oxidase assays were performed to determine fundamental physiological traits. Colony morphology and cellular structure were examined using both macroscopic and microscopic observations. Growth characteristics were also evaluated on yeast mannitol agar (YMA) supplemented with bromothymol blue to assess metabolic activity. Additionally, the strain was tested for tolerance to environmental stresses, including temperature

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and salinity, as these parameters are critical for selecting rhizosphere nitrogen-fixing bacteria (RNFB) suitable for degraded soil rehabilitation.

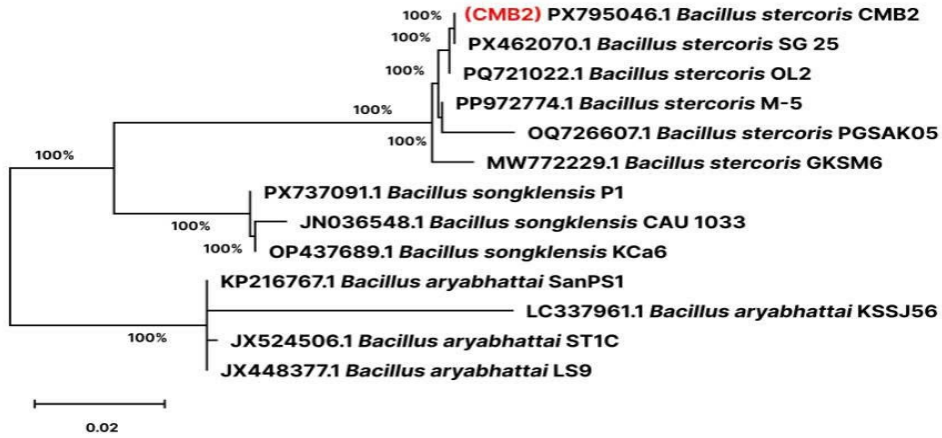

Figure 2. Phylogenetic relationships inferred from 16S rRNA gene sequences demonstrating the taxonomic placement of *B. stercoris* CMB2 within the *Bacillus stercoris* clade. The isolate showed 100% sequence similarity to *Bacillus stercoris* SG25. Bootstrap support values (%) are indicated at the corresponding branch nodes.

3. Results

3.1. Evaluation of Nitrogen Fixation Capacity

Twenty-five baby maize root samples were collected from Cho Moi Commune, An Giang Province, during the vegetative growth stage. Sampling targeted the rhizosphere zone by collecting root segments along with the closely adhering soil, which represents a biologically active interface characterized by intense root–microbe interactions. Compared with bulk soil, rhizosphere soil typically harbors a denser and more metabolically active microbial community. Immediately after collection, samples were transported under refrigerated conditions and processed within 24 h to preserve the integrity of the native microbial populations and minimize community shifts. Serial dilution and culture-based isolation techniques were applied, resulting in ten morphologically distinct bacterial colonies, designated CMB1–CMB10. Preliminary identification included Gram staining and detailed morphological examination, and the corresponding characteristics are presented in Table 1.

Table 1. Identification of 10 selected colonies from baby maize roots.

Strains	Identity (rod shape), gram	YMA (Clear pink)	YMA-BTB (Yellow color)	GPA	Hofer agar	Burk Agar
CMB1	(+)	(+),	(+)	(+)	(-)	(+)
CMB2	(++)	(++)	(++)	(++)	(-)	(-)
CMB3	(+)	(+)	(+)	(+)	(-)	(-)
CMB4	(+)	(+)	(+)	(+)	(-)	(++)
CMB5	(+)	(+)	(+)	(+)	(-)	(+)
CMB6	(+)	(+)	(+)	(+)	(-)	(+)
CMB7	(+)	(+)	(+)	(+)	(-)	(-)
CMB8	(+)	(+)	(+)	(+)	(-)	(-)
CMB9	(+)	(+)	(+)	(+)	(-)	(-)
CMB10	(+)	(+)	(+)	(+)	(-)	(-)

Note: (-) negative gram; (-): no reaction; (++); strong reaction.

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The comparative evaluation of morphological and biochemical traits (Tables 1 and 2) revealed clear differences among the ten isolates. All strains exhibited rod-shaped, Gram-positive characteristics and typical growth on YMA; however, only CMB2 showed consistently strong reactions (++) across most screening media, including YMA, YMA-BTB, and GPA. Notably, CMB4 demonstrated the highest growth intensity (++) on nitrogen-free Burk agar, suggesting strong diazotrophic potential, whereas most other isolates showed weak or no growth. Biochemical profiling further distinguished CMB2 as metabolically the most active strain, presenting strong (++) oxidase, catalase, urea hydrolysis, nitrate reduction, and citrate utilization activities. Although CMB4 exhibited superior qualitative growth on nitrogen-free medium, the overall physiological robustness and enzymatic versatility of CMB2 indicate a broader metabolic capacity. Considering both nitrogen-free growth and biochemical performance, CMB4 appears to possess the strongest direct nitrogen-fixing capability, while CMB2 demonstrates the highest overall functional potential.

Table 2. Biochemical tests of 10 selected colonies.

Strains	Oxidase	Catalase	Urea hydrolysis	Nitrate reduction	Citrate utilization
CMB1	(+)	(+)	(+)	(-)	(+)
CMB2	(++)	(++)	(++)	(++)	(++)
CMB3	(++)	(+)	(++)	(+)	(+)
CMB4	(+)	(+)	(+)	(+)	(+)
CMB5	(+)	(+)	(+)	(+)	(+)
CMB6	(+)	(+)	(+)	(+)	(+)
CMB7	(+)	(+)	(+)	(+)	(+)
CMB8	(+)	(+)	(+)	(+)	(+)
CMB9	(+)	(+)	(+)	(+)	(+)
CMB10	(+)	(+)	(+)	(+)	(-)

Note:(-): no reaction; (+); reaction.

The comparative tolerance analysis presented in Table 3 reveals clear differences in environmental adaptability among the ten isolates. All strains exhibited strong growth (++) at 1–3% NaCl and optimal temperatures of 37–40 °C, confirming their mesophilic and moderately halotolerant nature. However, CMB2 demonstrated superior resilience by maintaining vigorous growth (++) at 4% NaCl and sustaining positive growth even at 5%, whereas most other strains showed reduced growth or inhibition at higher salinity levels. In terms of temperature tolerance, CMB2 was the only strain exhibiting strong growth across the full tested range, including 15 °C and 45 °C. Regarding pH adaptability, while most isolates performed optimally at pH 6.0–7.0, CMB2 maintained consistent strong growth (++) across pH 5.0–8.0. Considering its broad tolerance to salinity, temperature, and pH stresses conditions that directly influence nitrogenase activity CMB2 appears to possess the strongest potential for stable nitrogen fixation under variable environmental conditions.

Table 3. Tolerance of ten microbial strains (V1-V10) to vary NaCl concentrations (%), temperatures (°C), and pH levels.

Strains	Nacl (%)					Temperature (°C)				pH			
	1	2	3	4	5	15	37	40	45	5.0	6.0	7.0	8.0
CMB1	++	++	++	+	-	+	++	++	+	++	++	++	+
CMB2	++	++	++	++	+	++	++	++	++	++	++	++	++
CMB3	++	++	++	+	-	++	++	++	+	++	++	++	+
CMB4	++	++	++	+	-	-	++	++	+	+	++	++	+
CMB5	++	++	++	+	-	+	++	++	+	++	++	++	+
CMB6	++	++	++	+	-	-	++	++	+	-	++	++	+
CMB7	++	++	++	+	-	+	++	++	+	+	++	++	+

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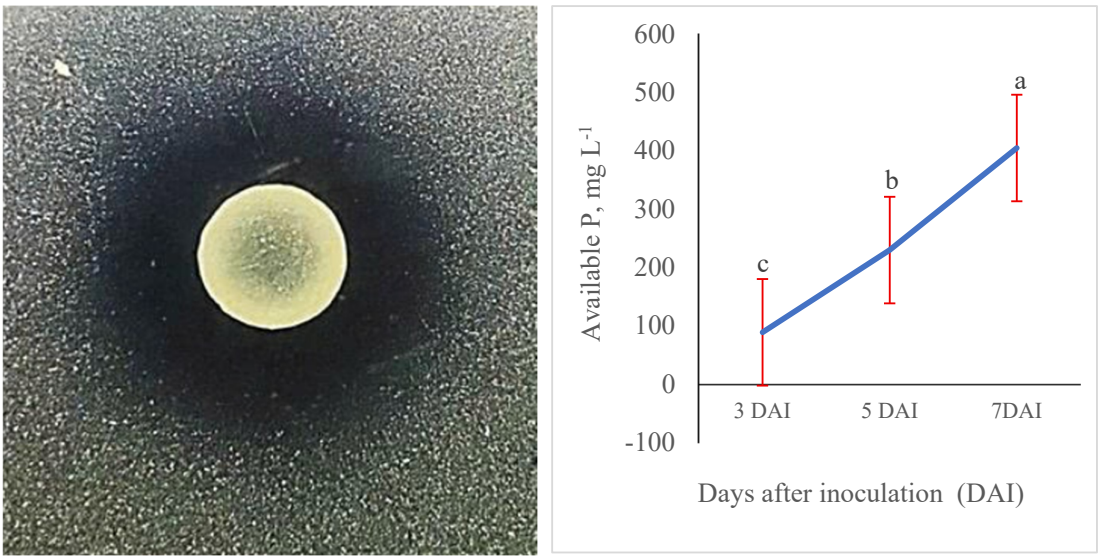
CMB8	++	++	++	+	+	+	++	++	+	+	++	++	+
CMB9	++	++	++	+	-	-	++	++	+	-	++	++	+
CMB10	++	++	++	+	-	+	++	++	+	++	++	++	+

The phylogenetic tree (Figure 2) demonstrates that analysis based on 16S rRNA gene sequences robustly positioned strain *Bacillus stercoris* CMB2 within the *Bacillus stercoris* clade. The isolate exhibited 100% sequence similarity to *B. stercoris* strain SG25, exceeding the widely accepted threshold for species-level identification. High bootstrap values at the corresponding nodes further support the reliability and stability of this phylogenetic placement. In comparison with closely related taxa within the genus Bacillus, *stercoris* species and strain CMB2 clustered tightly with reference sequences of *B. stercoris*, indicating strong genetic relatedness and minimal sequence divergence. Collectively, these molecular findings substantiate the accurate taxonomic assignment of *B. stercoris* CMB2 and corroborate the previously observed morphological and biochemical characteristics.

3.2. Assessment of Phosphate-Solubilizing Capacity

Ten rhizospheric bacterial isolates, selected after evaluating their nitrogen-fixing capacity and designated CBM1 to CBM10, were further assessed for their phosphate-solubilizing ability (PSB). The isolates were analyzed over a period of 7 days based on the diameter of the phosphate-solubilization halo. After 7 days of incubation, only strain CBM2 produced a clear solubilization halo, with a diameter of 24.6 mm and a colony diameter of 7.2 mm, corresponding to a D/d value of 3.42 (Figure 3).



(a) (b)

Figure 3. Phosphate-solubilizing activity of *B. stercoris* CMB2 on NBRIP agar showing a clear halo zone (a) and soluble phosphorus concentration at 3, 5, and 7 days of incubation (b).

Figure 3 illustrates the phosphate-solubilizing capacity of strain CMB2 on NBRIP medium. The formation of a distinct halo zone surrounding bacterial colonies confirms the ability of *B. stercoris* CMB2 to mobilize insoluble phosphate, indicating active secretion of solubilizing agents. Quantitative analysis further revealed a time-dependent increase in soluble phosphorus concentration, with values progressively rising from day 3 to day 7 of incubation. The gradual enhancement suggests sustained metabolic activity and continuous release of organic acids or phosphatases into the medium. The highest soluble phosphorus level recorded on day 7 indicates that phosphate solubilization efficiency improves during the late exponential phase of growth. This pattern demonstrates both qualitative (halo formation) and quantitative (P concentration) evidence

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of effective phosphate solubilization. Compared with typical phosphate-solubilizing bacteria, the progressive increase over time suggests strong enzymatic activity and metabolic stability of *B. stercoris* CMB2 under in vitro conditions.

3.3. Assessment of Nitrogen Fixation Capacity of B. stercoris CMB2

Figure 4 illustrates the nitrogen accumulation dynamics of *B. stercoris* CMB2 over time. Nitrogenase activity increased progressively during the early incubation period, reaching a peak at the mid-logarithmic phase, followed by a gradual decline at later stages. A parallel trend was observed for total nitrogen concentration in the culture medium, indicating a positive correlation between enzymatic activity and nitrogen fixation efficiency. The highest nitrogenase activity coincided with maximal metabolic growth, suggesting optimal energy availability for the nitrogenase complex. The subsequent decrease is likely to reflect oxygen sensitivity and feedback regulation associated with accumulated nitrogen compounds.

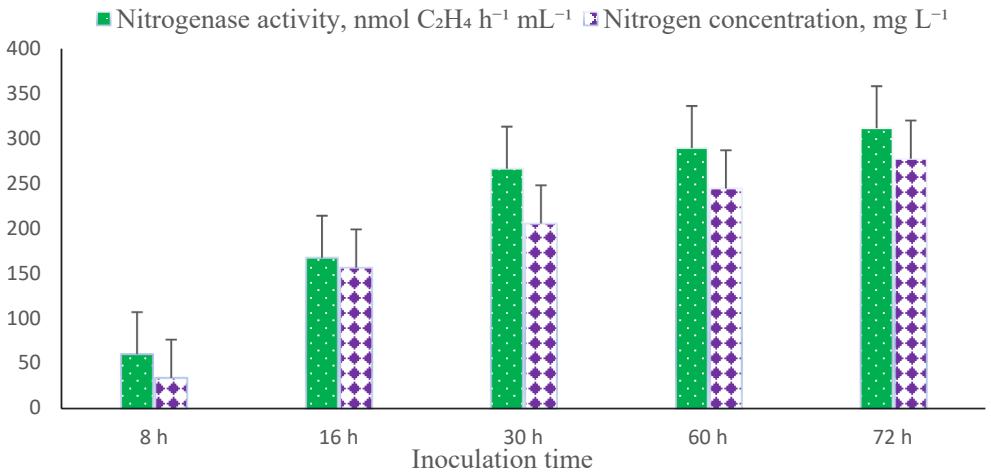

Figure 4. Nitrogenase activity (nmol C_2H_4 h^{-1} mL^{-1}) and total nitrogen concentration (mg L^{-1}) of *B. stercoris* CMB2 during incubation.

4. Discussion

4.1. Isolation and Morphophysiological Characterization of B. stercoris CMB2

The ability of bacterial isolates to grow vigorously on nitrogen-free media remains a fundamental screening criterion for identifying putative diazotrophs, as sustained colony development reflects the presence of an active nitrogenase complex capable of atmospheric N_2 reduction [46]. Recent investigations confirm that isolates demonstrating robust proliferation on Burk's or Ashby's media frequently exhibit high nitrogenase activity when quantified using acetylene reduction or total nitrogen assays, thereby validating the reliability of this preliminary phenotypic selection strategy [47,48]. In the present study, the selected strains displayed intense growth under nitrogen-deficient conditions, indicating strong metabolic adaptation to N limitation and suggesting effective biological nitrogen fixation potential. Beyond diazotrophic capacity, oxidative stress tolerance is increasingly recognized as a determinant of rhizosphere competence. Elevated catalase and oxidase activities, as observed in *B. stercoris* CMB2, likely enhance detoxification of reactive oxygen species generated during root colonization and fluctuating soil redox conditions. Recent studies emphasize that antioxidant enzyme systems are closely associated with improved survival, root adherence, and persistence of plant growth promoting bacteria under field stresses, ultimately sustaining nitrogenase functionality [48,49]. The concurrence of diazotrophic growth and strong

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oxidative defense mechanisms therefore supports the classification of *B. stercoris* CMB2 as a physiologically resilient candidate for bioinoculant development [50].

Environmental adaptability further strengthens its agronomic relevance. Salinity tolerance up to moderate NaCl concentrations and growth across a broad pH and temperature spectrum suggest metabolic plasticity and enzyme stability under abiotic stress [51]. Recent evidence indicates that diazotrophs capable of tolerating 3–5% NaCl and temperature fluctuations maintain more stable nitrogenase activity and rhizosphere colonization efficiency, particularly in marginal soils affected by salinization and climate variability [18,24,27]. pH adaptability is equally important, as nitrogenase expression and energy metabolism are sensitive to cytoplasmic pH shifts. Strains maintaining growth under variable pH conditions are more likely to sustain ATP generation and reductant supply required for N_2 fixation [18,24,27]. Collectively, the physiological robustness observed here aligns with current criteria for selecting high-performance nitrogen-fixing inoculants suited to diverse agroecological environments. Molecular identification based on 16S rRNA gene sequencing revealed ≥98.65% similarity with reference strains, exceeding the widely accepted threshold for species delineation. Contemporary taxonomic frameworks continue to endorse this cutoff for preliminary identification, although genome-based metrics such as average nucleotide identity (ANI) are recommended for resolving closely related taxa within the genus Bacillus and allied groups [18,20,24,27]. Nonetheless, the observed 100% sequence similarity strongly supports species-level assignment and provides a reliable phylogenetic basis for subsequent functional characterization.

4.2. Evaluation of Phosphate Solubilization Potential and Nitrogen Fixation Capacity of B. stercoris CMB2

The formation of clear halos on NBRIP agar and the progressive increase in soluble phosphorus concentration over time indicate efficient mineral phosphate solubilization. This phenotype is typically attributed to the secretion of low-molecular-weight organic acids such as gluconic, citric, or oxalic acids which chelate cations bound to insoluble phosphates and lower the surrounding pH, thereby mobilizing P into bioavailable forms. Recent studies confirm that peak phosphate solubilization commonly coincides with the exponential growth phase, when carbon metabolism and organic acid production are most active [52]. The sustained increase in soluble P up to day 7 observed in *B. stercoris* CMB2 suggests stable metabolic activity rather than transient enzymatic release, highlighting its suitability for prolonged rhizosphere functioning. Comparable solubilization kinetics have been documented in efficient strains of Bacillus and related genera applied as biofertilizers in sustainable cropping systems [53]. The gradual yet consistent P release pattern is agronomically advantageous, as it aligns nutrient availability with plant demand while minimizing fixation losses in soil matrices. Moreover, simultaneous nitrogen fixation and phosphate mobilization may generate synergistic effects on plant nutrition, enhancing both N and P uptake efficiency and promoting root development [54].

Nitrogenase activity profiling revealed a peak during the exponential phase, followed by a decline in stationary growth. This trend is widely reported in diazotrophic bacteria and is mechanistically linked to increased ATP availability and reductant flux during active cell division [31,55,56]. As cultures transition to stationary phase, oxygen sensitivity of nitrogenase and regulatory feedback mechanisms typically suppress enzyme synthesis, leading to reduced activity [57]. The nitrogenase activity pattern observed in *B. stercoris*, with a maximum during exponential growth followed by decline in the stationary phase, aligns with established regulatory models of biological nitrogen fixation. Nitrogenase expression is closely linked to ATP availability, oxygen sensitivity, and feedback inhibition by fixed nitrogen, as recently discussed in Nitrogen [57,58]. Beyond *B. stercoris*, the strain exhibited oxidative stress tolerance, environmental adaptability, and effective phosphate solubilization. The integration of these complementary traits highlights the multifunctional potential of CMB2 as a next-generation biofertilizer to enhance nutrient-use efficiency and reduce dependence on synthetic fertilizers in sustainable agricultural systems [59,60].

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5. Conclusions

This study successfully isolated and characterized a multifunctional endophytic bacterium, *Bacillus stercoris* CMB2, from baby maize roots cultivated in the Mekong Delta of Vietnam. The strain exhibited strong growth on nitrogen-free media, significant nitrogenase activity, and a consistent increase in total nitrogen accumulation, confirming its effective biological nitrogen fixation capacity. Notably, *B. stercoris* CMB2 was the only isolate among ten candidates demonstrating pronounced phosphate-solubilizing ability, evidenced by clear halo formation on NBRIP agar and a progressive rise in soluble phosphorus concentration over time. The strain also showed broad tolerance to salinity (up to 5% NaCl), temperature (15–45 °C), and pH (5.0–8.0), indicating high environmental adaptability. The key novelty of this work lies in the identification of a locally adapted *B. stercoris* CMB2 simultaneously possessing strong nitrogen-fixing and phosphate-solubilizing capacities from baby maize tissues in Vietnam. This dual-functional trait, combined with stress resilience, highlights its promising potential as a sustainable biofertilizer candidate to improve nutrient-use efficiency and reduce chemical fertilizer dependency in baby maize production systems.

Data Availability: The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: There is no conflict of interest among the authors.

Authors' Contributions: Tran Thanh Liem collected samples and contributed all research funds and wrote down the whole manuscript; Tran Thanh Liem and Nguyen Van Chuong carried out the laboratory work (both isolation and molecular identification). We approved the final version of this manuscript.

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References

1. Aarizoo M.; Abida, J.; Monisa, Y.; Mudasir, A. M.; Taha, M.; Tawheed, A.; Shabnam, S.; Asima, R. Post-Harvest Quality Management of Sweet Corn: Disorders, Losses and Preservation Strategies. *Food Nutrition* **2025**, 1(1), 100007.
2. Sidahmed, H.; Vad, A.; Nagy, J. Advances in Sweet Corn (*Zea mays* L. saccharata) Research from 2010 to 2025: Genetics, Agronomy, and Sustainable Production. *Agronomy* **2025,** 15, 1260.
3. Phuong, T.T.; Vien, T.D.; Son, C.T.; Thuy, D.T.; Greiving, S. Impact of Climate Change on Agricultural Production and Food Security: A Case Study in the Mekong River Delta of Vietnam. *Sustainability* **2024**, 16, 7776.
4. Kirui, E.C.; Kidoido, M.M.; Akutse, K.S.; Wanyama, R.; Boni, S.B.; Dubois, T.; Dinssa, F.F.; Mutyambai, D.M. Factors Influencing the Adoption of Agroecological Vegetable Cropping Systems by Smallholder Farmers in Tanzania. *Sustainability* **2025**, 17, 1148.
5. Liu, Z.; Guo, Z.; Wang, Z.; Xie, W.; Yang, Z.; He, L.; Chen, D.; Wu, X.; Zhou, H. Response of Maize Yield and Water Productivity to Different Long-Term Fertilization Strategies in Semi-arid Regions in Northern China. *Agriculture* **2025,** 15, 2396.
6. Rajan, B.; Kunal, D.M.; Viliam, B.; Marian, B.; Ahmed, G.; Milan, S.; Akbar, H. Sustainable Strategies to Limit Nitrogen Loss in Agriculture Through Improving Its Use Efficiency Aiming To Reduce Environmental Pollution. J. Agric. *Food Res.* **2025**, 22, 101957.
7. Manono, B.O.; Kimiti, J.M.; Musyoka, D.K. Landscape Determinants of Nitrogen Leaching Risk: Mechanisms, Impacts, and Mitigation Strategies. *Nitrogen* **2026**, 7, 20.
8. Liu, J.; Yu, Z.; Gong, Y.; Chen, J.; Zhou, L.; Zhang, W.; Jia, L. Fertilization Induced Soil Microbial Shifts Show Minor Effects on Sapindus mukorossi Yield. *Microorganisms* **2025**, 13, 173.
9. Van Chuong, N.; Tri, T.L.K.; Vu, T.M.; Tuan LM, Liem TT, Trang NNP. Isolation and Identification of *Bacillus aryabhattai* M2C: Its Effects with Vermicompost on Yield and Nutrients of Peanut (*Arachis hypogaea* L.). *Int J Microbiol.* **2025,** 10, 9923279.

 doi:10.20944/preprints202602.1520.v1

10. Qui, N.V.; Khoa, L.V.; Phuong, N.M.; Vien, D.M.; Dung, T.V.; Linh, T.B.; Khanh, T.H.; Thuong, B.T.; Tran, V.T.T.; Nghia, N.K.; et al. Effects of Rotating Rice with Upland Crops and Adding Organic Amendments, and of Related Soil Quality on Rice Yield in the Vietnamese Mekong Delta. *Agronomy* **2024**, 14, 1185.
11. Sridhar, R.; Longkumer, L.T.; Pilla, A.; Bharteey, P.K.; Jatav, H.S.; Hareesh, D.; Vilakar, K.; Singh, A.P.; Srikar, K.; Aruna, K.; Paarshitha Reddy, M.S. Diverse Fields for Stronger Yields: Crop Diversification Strategies for Sustainable Agriculture and Climate-Resilient Ecosystems. *Front. Agron.* **2026**, 7, 1746895.
12. Qiang, W.; Ai, P.; Ma, Y.; Zhao, J. The Impact of Water Deficit at Various Growth Stages on Physiological Characteristics, Fruit Yield, and Quality of Drip-Irrigated Jujube Trees. *Agronomy* **2025**, 15, 1205.
13. Pritha, K.; Saikat, B. Securing Soil Health as the Foundation for Strengthening Planetary Health, Soil Security. *Soil Secur.* 2025, 7, 1746895.
14. Díaz-Rodríguez, A.M.; Parra Cota, F.I.; Cira Chávez, L.A.; García Ortega, L.F.; Estrada Alvarado, M.I.; Santoyo, G.; de los Santos-Villalobos, S. Microbial Inoculants in Sustainable Agriculture: Advancements, Challenges, and Future Directions. *Plants* **2025**, 14, 191.
15. Van Chuong, N.; Thanh Liem, T.; Le Kim Tri, T.; Ngoc Phuong Trang, N.; Tran Hai Dang, P. Effects of *Bacillus songklensis* and *Bacillus siamensis* WD-32 Combined with Vermicompost on Soil Fertility, Growth, Yield, and Arsenic Accumulation in Peanut. *Asian J. Agric. Biol.* **2025**, e2025142.
16. Ansabayeva, A.; Makhambetov, M.; Rebouh, N.Y.; Abdelkader, M.; Saudy, H.S.; Hassan, K.M.; Nasser, M.A.; Ali, M.A.A.; Ebrahim, M. Plant Growth-Promoting Microbes for Resilient Farming Systems: Mitigating Environmental Stressors and Boosting Crops Productivity: A Review. *Horticulturae* **2025**, 11, 260.
17. Chuong, N.V.; Nguyen Ngoc Phuong, T.; Nguyen Van, T. Nitrogen Fertilizer Use Reduction by Two Endophytic Diazotrophic Bacteria for Soil Nutrients and Corn Yield. *CST* **2024**, *9*, 348-355
18. Chuong, N.V.; Vu, T.M.; Tuan, L.M.; Son, N.T.T.; Tri, T.L.K.; Thuan, N.V.; Dang, P.T.H.; Liem, T.T.; Trang, N.N.P. Enhanced Soil Fertility and Baby Maize Yield Through *Bacillus Megaterium* CM2 under Reduced Nitrogen Input. *CST* **2025**, 10, 411-421.
19. Bolla, P.K.; Panozzo, A.; Minozzi, E.; Valente, F.; Potestio, S.; Visioli, G.; Martinez-Sañudo, I.; Vamerali, T. Effects of Foliar-Sprayed Bio-Fertilizer with N-Fixing *Methylobacterium symbioticum* On Morpho-Physiological Traits of Maize Under Varying N Fertilization Rates. *Front Plant Sci.* **2025,** 4;16,1661290.
20. Nguyen, V.C.; Nguyen, N.P. T.; Tran, T.L.; Phan, T.H.D. Effect of *Bacillus sonklengsis* Associated with Cattle Manure Fertilization on the Farmland Health and Peanut Yield. *Int. J. Agric. Biosci.* **2025** 14(4), 629-636
21. Pan, L.; Cai, B. Phosphate-Solubilizing Bacteria: Advances in Their Physiology, Molecular Mechanisms and Microbial Community Effects. *Microorganisms* **2023,** 11(12),2904.
22. Mamo, B.; Wubayehu, G. Phosphate Solubilizing Bacteria in Optimizing Phosphorous Acquisition and Crop Production. *Am. J. BioScience* **2022**, 10(2), 61-68.
23. Pan, L.; Cai, B. Phosphate-Solubilizing Bacteria: Advances in Their Physiology, Molecular Mechanisms and Microbial Community Effects. *Microorganisms* **2023**, 11(12),2904.
24. Chuong, N.V.; Tri, T.L.K.; Liem, T.T.; Thuan, N.V., Em, T.V.T.; Trang, N.N.P. Four Newly Identified Endophytic *Bacillus megaterium* Strains with Biofertilizer Potential for Enhancing Maize Growth and Productivity. *TiS.* 2026, 23(3), 11347.
25. Ramírez-López, M.; Bautista-Cruz, A.; Toledo-López, A.; Aquino-Bolaños, T. Rhizospheric and Endophytic Plant Growth-Promoting Bacteria Associated with *Coffea arabica* L. and *Coffea canephora* Pierre ex Froehner: A Review of Their Agronomic Potential. *Microorganisms* **2025** 3(11), 2567.
26. Díaz-Rodríguez, A.M.; Parra Cota, F.I.; Cira Chávez, L.A.; García Ortega, L.F.; Estrada Alvarado, M.I.; Santoyo, G.; de Los Santos-Villalobos, S. Microbial Inoculants in Sustainable Agriculture: Advancements, Challenges, and Future Directions. *Plants (Basel)* **2025**,14(2),191.
27. Van Chuong, N.; Le Kim Tri, T. Isolation and Characterization Identification of Edophytic Nitrogen-Fixing Bacteria from Peanut Nodules. *Int. J. Microbiol.* **2024**, 8973718, 1-7.
28. 28. Wekesa, C.; Furch, A.C.U.; Ralf, O. Isolation and Characterization of High-Efficiency Rhizobia from Western Kenya Nodulating with Common Bean. *Front. Microbiol.* **2021**, 10(12), 697567.
29. Somasegaran, P.; Hoben, H.J. Methods in Legume-Rhizobium Technology. NifTAL Project and MIRCEN. Department of Agronomy, 2nd Soil Science Hawaii Institute Tropical Agriculture Human Research, University of Hawaii at Manoa, Honolulu, **1985**.

 doi:10.20944/preprints202602.1520.v1

30. Davis, K.E.; Joseph, S.J.; Janssen, P.H. Effects of Growth Medium, Inoculum Size, and Incubation Time on Culturability and Isolation of Soil Bacteria. *Appl Environ Microbiol*. **2005**, 71(2), 826-834
31. Van Chuong, N. The impact of *Klebsiella quasipneumoniae* inoculation with nitrogen fertilization on baby corn yield and cob quality. *Eurasian J. Soil Sci.* **2024**, 13(2), 133-138.
32. Agunbiade, V.F.; Fadiji, A.E.; Agbodjato, N.A.; Babalola, O.O. Isolation and Characterization of Plant-Growth-Promoting, Drought-Tolerant Rhizobacteria for Improved Maize Productivity. *Plants* **2024**, 13, 1298.
33. Nguyen, V.C. Influences of *Enterobacter asburiae*, Vermicompost Rates and Irrigation Water Types on the Soil Fertility, Peanut Yield and Quality. *Curr. Appl. Sci. Technol*. **2025**, 25(4), e0260428.
34. Sanders, E.R. Aseptic Laboratory Techniques: Plating Methods. *J Vis Exp.* **2012**,11(63),e3064.
35. Sharma, A.; Lomba, P.S.; Mishra, B.; Bhasin, A.; Reshi, S.; Diagnostic Significance of Wet Mount Microscopy- A Retrospective Observational Study. *J Clin of Diagn Res* .**2022**, 16(3), DC14-DC19.
36. Osiro, D.; Filho, R.B.; Assis, O.B.; Jorge, L.A.; Colnago, L.A. Measuring Bacterial Cells Size with AFM. *Braz J Microbiol.* **2012**,43(1), 341-347.
37. Tripathi, N.; Zubair, M.; Sapra, A. Gram Staining. [Updated 2025 Mar 28]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; **2025** Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK562156/.
38. Palma, V.; Gutiérrez, M.S.; Vargas, O.; Parthasarathy, R.; Navarrete, P. Methods to Evaluate Bacterial Motility and Its Role in Bacterial-Host Interactions. *Microorganisms* **2022** 10(3),563.
39. Iwase, T.; Tajima, A.; Sugimoto, S.; Okuda, K.; Hironaka, I.; Kamata, Y.; Takada, K.; Mizunoe, Y. A Simple Assay for Measuring Catalase Activity: A Visual Approach. *Sci Rep.* **2013,** 3:3081.
40. Fulweiler, R.W.; Heiss, E.M.; Rogener, M.K.; Newell, S.E.; LeCleir, G.R.; Kortebein, S.M.; Wilhelm, S.W. Examining The Impact of Acetylene On N-Fixation and The Active Sediment Microbial Community. *Front. Microbiol.* 2015; 6, 418
41. Kifle, M.H., Laing, M.D. Isolation and Screening of Bacteria for Their Diazotrophic Potential and Their Influence on Growth Promotion of Maize Seedlings in Greenhouses. *Front Plant Sci.* **2016**, 8;6,1225.
42. Santos, J.; Cardoso, P.; Rocha, R.; Pinto, R.; Lopes, T.; Patinha, C.; Guilherme, R.; Ferreira, A.; Figueira, E. Phosphate-Solubilizing Bacteria from Different Genera, Host Plants, and Climates: Influence of Soil pH on Plant Growth and Biochemistry. *Land* **2025**, 14, 2065.
43. Aliyat, F.Z.; Maldani, M.; El Guilli, M.; Nassiri, L.; Ibijbijen, J. Phosphate-Solubilizing Bacteria Isolated from Phosphate Solid Sludge and Their Ability to Solubilize Three Inorganic Phosphate Forms: Calcium, Iron, and Aluminum Phosphates. *Microorganisms* **2022**, 10, 980.
44. Edi Premono, M.; Moawad, A.M.; Vlek, P.L.G. Effect of Phosphate-Solublizing Pseudomonas Putida on The Growth of Maize and Its Survival in The Rhizosphere. *Indonasian J Crop Sci.* **1996**,11,13–23.
45. Berza, B.; Sekar, J.; Vaiyapuri, P.; Pagano, M.C.; Assefa, F. Evaluation of Inorganic Phosphate Solubilizing Efficiency and Multiple Plant Growth Promoting Properties of Endophytic Bacteria Isolated from Root Nodules Erythrina Brucei. *BMC Microbiol.* **2022**, 22(1),276.
46. Koirala, A.; Alshibli, N.A.; Das, B.K.; Brözel, V.S. Bacterial Isolation from Natural Grassland on Nitrogen-Free Agar Yields Many Strains Without Nitrogenase. *Microorganisms* **2025**, 13, 96.
47. Haskett, T.L.; Knights, H.E.; Jorrin, B.; Mendes, M.D.; Poole, P.S. A Simple in situ Assay to Assess Plant-Associative Bacterial Nitrogenase Activity. *Front Microbiol.* **2021**, 12,690439.
48. Singh, R.K.; Singh, P.; Sharma, A.; Guo, D.-J.; Upadhyay, S.K.; Song, Q.-Q.; Verma, K.K.; Li, D.-P.; Malviya, M.K.; Song, X.-P.; et al. Unraveling Nitrogen Fixing Potential of Endophytic Diazotrophs of Different Saccharum Species for Sustainable Sugarcane Growth. *Int. J. Mol. Sci.* **2022**, 23, 6242.
49. Imran, A.; Hakim, S.; Tariq M, Nawaz MS, Laraib I, Gulzar U, Hanif MK, Siddique MJ, Hayat M, Fraz A, Ahmad M. Diazotrophs for Lowering Nitrogen Pollution Crises: Looking Deep into the Roots. *Front Microbiol*. **2021** ,12,637815.
50. Pankievicz, V.C.S.; do Amaral, F.P.; Ané, J.M.; Stacey, G. Diazotrophic Bacteria and Their Mechanisms to Interact and Benefit Cereals. *MPMI* **2021**, 34(5), 491–498.

 doi:10.20944/preprints202602.1520.v1

51. Susanna, C.; Giulia, C.; Rita, M.; Stefano, B.; Antonio, F.; Luca, I.; Anna, M.; Alice, T. Physiological and Hormonal Responses Underlying Salinity Tolerance In Wild Tomatoes: Insights For Cultivated Varieties. *Environ. Exp. Bot.* **2026**, 241,106296.
52. Teles, E.A.P.; Xavier, J.F.; Arcenio, F.S.; Amaya, R.L.; Gonc¸alves, J.V.S. Rouws, L.F.M.; Zonta, E.; Coelho, I.S. Characterization And Evaluation of Potential Halotolerant Phosphate Solubilizing Bacteria from Salicornia Fruticosa Rhizosphere. *Front. Plant Sci.* **2024**,14,1324056.
53. Thaqi, S.K.; Siani, R.; Chiba, A.; Peine, M.; Baum, C.; Witting, M.; Walch, S.; Leinweber, P.; Schloter, M.; Schulz, S. Strain-Specific Strategies Underlie Convergent Phosphate Solubilization in *Bacillus*. *ISME Commun.* **2025**, 5(1), ycaf208.
54. Peng, L.; Yiming, W.; Yazhuo, W.; Fei, T.; Zhansheng, W. Biochar-Based Slow-Release Fertilizers: From Nutrient Carrier to Intelligent Regulator in Soil-Plant Systems-A Review and Perspective. *J. Environ. Chem. Eng.* **2026**, 14, 121720,
55. Liu, X.; Li, P.; Bao, K.; Wang, Y.; Wang, H.; Wang, Y.; Jiang, Z.; Yang, Y.; Yuan, S.; Kappler, A.; Wang, Y. Synergistic Interaction Between Microbial Nitrogen Fixation And Iron Reduction In The Environment. *ISME J.* **2025** 19(1), wraf212.
56. Chuong, N.V. The Impact of *Bacillus* sp. NTLG2-20 and Reduced Nitrogen Fertilization on Soil Properties and Peanut Yield. *CST* **2024**, *9*, 112-120.
57. Nawaz, T.; Fahad, S.; Gu, L.; Xu, L.; Zhou, R. Harnessing Nitrogen-Fixing Cyanobacteria for Sustainable Agriculture: Opportunities, Challenges, and Implications for Food Security. *Nitrogen* **2025**, 6, 16.
58. Herrero, J.; Ramírez-Santos, A.; Díaz-Santos, E.; Torres-Cortés, G. Biofertilizers for Enhanced Nitrogen Use Efficiency: Mechanisms, Innovations, and Challenges. *Nitrogen* **2025**, 6, 111.
59. Ughamba, K.T.; Ndukwe, J.K.; Lidbury, I.D.E.A.; Nnaji, N.D.; Eze, C.N.; Aduba, C.C.; Groenhof, S.; Chukwu, K.O.; Anyanwu, C.U.; Nwaiwu, O.; et al. Trends in the Application of Phosphate-Solubilizing Microbes as Biofertilizers: Implications for Soil Improvement. *Soil Syst.* 2025, 9, 6.
60. Bansal, R., Vyas, P., Sharma, S. Formulation and field performance of liquid phosphate-solubilizing bacterial biofertilizer for improved growth, yield, and nutrient content of potato. *Front. Microbiol.* **2025,** *16*, 1734471.