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Complementary nutrient-mobilizing traits in herbal rhizosphere bacteria support a multi-strain bioinoculant

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Abstract

Excessive use of synthetic fertilizers has driven interest in microbial alternatives for sustainable agriculture, and herbal plant rhizospheres host distinctive microbial communities with bio-fertilization potential. Eleven bacterial isolates representing nine species (*Priestia megaterium*, *Priestia aryabhattai*, *Bacillus cereus*, *Bacillus xiamenensis*, *Enterobacter chuandaensis*, *Enterobacter cloacae*, *Burkholderia seminalis*, *Burkholderia multivorans* and *Chromobacterium violaceum*) and three *Trichoderma* endophytes (two *T. yunnanense* and one *T. simmonsii*) were isolated from the rhizosphere of *Barleria lupulina*. They were identified by 16S rRNA or internal transcribed spacer (ITS) gene sequencing, sequence similarity searches and, for the bacteria, phylogenetic analysis. Growth on nitrogen-free medium was widespread, and all 14 isolates produced ammonia. Phosphate and potassium solubilization were restricted to *B. seminalis* and *B. multivorans*, with solubilization indices of 2.45 and 2.16 on Pikovskaya agar and 2.38 and 2.83 on Aleksandrov agar, respectively. Zinc solubilization occurred in *B. cereus*, *E. chuandaensis*, *E. cloacae* and *B. xiamenensis* (indices 2.09–2.57). As no single isolate showed all four traits, a three-strain consortium (*E. chuandaensis*, *B. seminalis* and *E. cloacae*) was selected to cover nitrogen fixation and phosphate, potassium and zinc solubilization, with redundancy for zinc. Isolates with confirmed nitrogen fixation were prioritized over the higher-index but weakly nitrogen-fixing *B. cereus*. Herbal rhizospheres are therefore a source of multi-trait plant growth-promoting rhizobacteria (PGPR) for bioinoculant development.

Keywords: Bioinoculant; Rhizosphere; Herbal plants; Plant growth-promoting rhizobacteria (PGPR); NPK assay

1. Introduction

Agricultural productivity worldwide faces increasing challenges from soil degradation, chemical dependency, and environmental stress. The intensive use of synthetic fertilizers to sustain crop yields has contributed to soil acidification, nutrient leaching, reduced microbial diversity, and long-term ecosystem imbalance (*1*). As a result, there is growing interest in sustainable, biologically based strategies that can improve plant nutrition and soil health while reducing reliance on chemical inputs (*2*). In this context, plant growth-promoting rhizobacteria (PGPR) have emerged as promising eco-friendly agents for enhancing crop performance through a range of direct and indirect mechanisms (*3*).

The rhizosphere is a biologically active soil zone surrounding plant roots, where complex interactions occur among plants, microorganisms, and soil constituents. This environment is enriched by root exudates that support diverse microbial populations, including bacteria with beneficial traits for plant growth and soil fertility (*4*). Rhizosphere-associated microorganisms are widely recognized for their roles in nutrient cycling, suppression of phytopathogens, and enhancement of plant productivity, making them important resources for sustainable agriculture (*5*). Among these microorganisms, PGPR have received considerable attention because of their ability to improve nutrient acquisition and stimulate growth through nitrogen fixation, phosphate solubilization, potassium mobilization, phytohormone production, siderophore secretion, and antimicrobial activity (*6*).

Herbal and medicinal plants represent a particularly valuable and underexplored reservoir of beneficial rhizosphere microorganisms (*7*). The unique secondary metabolites and root exudates released by these plants can shape the composition and functional diversity of their associated microbiota, potentially enriching for bacterial strains with strong plant-growth-promoting traits and stress tolerance. Isolation of indigenous bacteria from the rhizosphere of herbal plants may therefore yield strains adapted to specific soil-plant environments and suitable for use in bioinoculant formulations as reported by *Zhang, Li (8*). Such microbial products are

increasingly being explored as environmentally friendly alternatives to synthetic fertilizers and as tools to support resilient cropping systems.

The development of an effective bioinoculant mixture requires preliminary screening and characterization of bacterial isolates to determine their suitability for agricultural application. Functional assays related to nitrogen, phosphorus, and potassium (NPK) are essential for identifying strains with plant nutrition potential. Nitrogen-fixing bacteria convert atmospheric nitrogen into bioavailable forms, while phosphate-solubilizing and potassium-mobilizing microbes transform insoluble mineral compounds into forms accessible to plants (*9*). In addition, laboratory analyses such as Gram staining and bacterial identification provide important morphological and taxonomic information that supports the selection of safe, compatible, and functionally effective strains for multi-strain inoculant development.

Therefore, the present study aimed to isolate bacteria from the rhizosphere of selected herbal plants and evaluate their potential as bioinoculant candidates through NPK-related assays, phosphate solubilization screening, Gram staining, and bacterial identification. The selected isolates may serve as promising microbial resources for the development of sustainable bioinoculant mixtures for agricultural applications.

2. Materials and Methods

2.1 Root collection

Root samples were collected from nursery-grown plants. A total of three root samples (namely *Barleria lupulina* and *Typhonium flagelliforme*) were collected for microbial and endophyte isolation. The nursery was located at the Agro-Biotechnology Institute (ABI), National Institutes of Biotechnology Malaysia (NIBM), Serdang, Selangor, Malaysia.

2.2 Isolation of root-associated microorganisms

Rhizosphere bacteria were isolated from plants that were carefully uprooted to keep the root system and surrounding soil intact. Loosely held bulk soil was removed by gentle hand shaking, leaving the thin layer (1-2 mm) of tightly adhering soil that defines the functional rhizosphere. Root segments carrying this soil were excised with sterile forceps and scissors and placed directly into sterile containers for processing. For

each sample, 0.1 g of root material was suspended in 9 mL of phosphate-buffered saline (PBS) and incubated at 37 °C for 1 h. A 100 µL aliquot of the suspension was then spread onto Luria-Bertani (LB) agar and incubated at 37 °C for 2-5 days. Individual colonies were purified by quadrant streaking, and the purified isolates were maintained by subculturing in vials. For endophytic fungal isolation, Healthy root tissues were harvested and rinsed thoroughly under running tap water to remove debris. The roots were cut into segments of approximately 0.5-1.0 cm and surface-sterilized to eliminate epiphytic microflora. The segments were immersed in 70% (*v/v*) ethanol for 1 min, then in sodium hypochlorite solution (2-3% available chlorine) for 3-5 min, and finally rinsed five times with sterile distilled water. Sterilization efficacy was verified by plating aliquots of the final rinse water onto Potato Dextrose Agar (PDA) as a negative control. The surface-sterilized segments were blotted dry on sterile filter paper, placed onto PDA plates supplemented with an antibacterial agent (e.g., chloramphenicol or streptomycin, 50–100 mg/L), and incubated at room temperature (25 ± 2 °C) for 5–7 days. Plates were monitored daily for fungal hyphae emerging from the cut root edges. Emerging colonies representing endophytic fungi were aseptically excised, transferred onto fresh PDA plates, and subcultured until axenic cultures were obtained. The isolates were subsequently submitted for identification.

2.3 Genomic DNA Extraction, PCR Amplification, and DNA Barcoding

Rhizosphere bacterial isolates and endophytic fungal isolates were identified by DNA barcoding. Genomic DNA (gDNA) was extracted at Apical Scientific Sdn Bhd [Seri Kembangan, Selangor, Malaysia] using extraction protocols suited to bacteria and fungi. Bacterial gDNA was extracted with the Presto™ Mini gDNA Bacteria Kit (Geneaid), and fungal gDNA with the Presto™ Mini gDNA Yeast Kit (Geneaid) or 1st BASE Lysis Buffers, following the manufacturers' instructions. For bacterial identification, the near full-length 16S rRNA gene (~1500 bp) was amplified using the universal primers 27F and 1492R. Each 25 µL polymerase chain reaction (PCR) mixture contained purified gDNA, 0.3 pmol of each primer, 400 µM dNTPs, 0.5 U of thermostable DNA polymerase, the supplied PCR buffer, and nuclease-free water. Thermal cycling consisted of an initial denaturation at 94 °C for 2 min, followed by 25 cycles of 98 °C for 10 s, 53 °C for 30 s, and 68 °C for 1 min. The purified PCR products were sequenced bidirectionally using the primers 785F and 907R. For fungal identification, the internal transcribed spacer (ITS) region was amplified using the

universal primers ITS1 and ITS4. Each 25 µL reaction mixture contained purified gDNA, 0.5 pmol of each primer, 200 µM dNTPs, 0.5 U of thermostable DNA polymerase, the supplied reaction buffer, and nuclease-free water. Thermal cycling consisted of an initial denaturation at 98 °C for 2 min, followed by 25 cycles of 98 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. The purified PCR products were sequenced bidirectionally using the primers M13F (-20) and M13R-PUC (-26). All sequencing reactions were performed with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Forward and reverse chromatograms were inspected, quality-trimmed, and assembled into consensus sequences. Sequence similarity was assessed using the Nucleotide Basic Local Alignment Search Tool (BLASTn) against the National Center for Biotechnology Information (NCBI) database, and the top match, percentage identity, and accession number were recorded for each isolate.

The 16S rRNA gene sequences of the bacterial isolates and their closest reference sequences retrieved from NCBI were aligned using [alignment method, e.g., MUSCLE or ClustalW]. A Neighbor-Joining tree [9] was constructed in Molecular Evolutionary Genetics Analysis (MEGA) X software [10] using the [substitution model, e.g., Kimura 2-parameter model]. Branch support was assessed by bootstrap analysis [11] with [number, e.g., 1000] replicates. Fungal isolates were identified solely by BLASTn similarity of their ITS sequences, and no phylogenetic tree was constructed for them.

For phylogenetic analysis, the 16S rRNA gene sequences of the bacterial isolates and their closest reference sequences retrieved from NCBI were aligned [Megablast (Highly similar sequences) and a Neighbor-Joining tree was constructed in Molecular Evolutionary Genetics Analysis (MEGA) X software using [Kimura 2-parameter (K2P)]. Branch support was assessed by bootstrap analysis with 1000 replicates. Fungal isolates were identified by BLASTn similarity of their ITS sequences only.

2.4 Screening for nitrogen-fixation ability

The bacterial isolates were screened for nitrogen-fixation ability on nitrogen-free Jensen medium agar. Each bacterial isolate was inoculated onto one Jensen medium plate using an inoculation loop, with three separate inoculation spots made for each

isolate on the same plate. The inoculated plates were incubated at 37 °C for 3-7 days. Following incubation, bacterial growth at each inoculation spot was examined and recorded, and an isolate was classified as positive or negative for nitrogen-fixation ability based on visible growth. The three spots were treated as technical replicates on the same plate and not as independent biological replicates.

2.5 Phosphate solubilization assay on Pikovskaya agar

Phosphate-solubilizing ability of bacterial isolates was screened using Pikovskaya's (PVK) agar medium containing tricalcium phosphate as the insoluble phosphate source. Pure cultures were spot-inoculated on PVK agar plates containing an insoluble phosphate source such as tricalcium phosphate and incubated at 37 °C for 7 days or longer until clear zones appeared. Isolates forming a clear halo around the colony were considered positive for phosphate solubilization. Colony diameter and halo zone diameter were measured in millimeters (*10*). For phosphorus solubilization on Pikovskaya agar, the solubilization index (SI) was calculated as:

$$\text{Phosphorus solubilization index} = \frac{\text{Halo zone diameter + Colony diameter}}{\text{Colony diameter}}$$

2.6 Potassium solubilization assay on Aleksandrov agar

The potassium-solubilizing ability of the bacterial isolates was screened on Aleksandrov agar containing potassium aluminosilicate as the insoluble potassium source. Pure bacterial isolates were spot-inoculated onto the plates and incubated at 37 °C for 3-7 days. Isolates producing a clear halo around the colony were considered positive for potassium solubilization (*11*). The colony diameter and halo zone diameter were measured to calculate the solubilization index as:

$$\text{Potassium solubilization index} = \frac{\text{Halo zone diameter + Colony diameter}}{\text{Colony diameter}}$$

2.7 Zinc solubilization screening assay

The zinc-solubilizing ability of the bacterial isolates was screened by the plate halo method on [Bunt and Rovira's agar / Tris-mineral agar] supplemented with 0.1% [ZnO / $ZnCO_3$ / zinc phosphate] as the insoluble zinc source. Bacterial isolates were spot-inoculated onto the plates and incubated at 37 °C for 2-7 days. Isolates producing a clear halo around the colony were considered zinc solubilizers. Isolates producing a

clear halo zone around the colony were considered zinc solubilizers (*12*). The colony diameter and halo zone diameter were measured, and the zinc solubilization index was calculated as:

$$\text{Zinc solubilization index} = \frac{\text{Halo zone diameter + Colony diameter}}{\text{Colony diameter}}$$

2.8 Ammonia production assay in peptone water

Ammonia production by the bacterial isolates was assessed in 1% (*w/v*) peptone water containing 0.5% sodium chloride. Freshly grown cultures were inoculated into test tubes containing 10 mL of sterile peptone water and incubated at 37 °C for 48h. After incubation, 0.5 mL of Nessler's reagent was added to each tube. An uninoculated peptone water tube as a negative control. The development of a yellow to brown color was taken as a positive result for ammonia production.

3. Results

3.1 Isolation of root-associated bacteria and fungal endophytes

Bacterial growth was observed on LB agar after incubation of the root suspensions at 37 °C for 2-5 days. Following quadrant streaking, 11 bacterial isolates were obtained from the root samples, representing nine bacterial species (Fig. 1). *Priestia megaterium* was identified from Bacteria 1 and Bacteria 2, while *Bacillus cereus* was identified from Bacteria 3 and Bacteria 4. The remaining bacterial isolates were identified as *Priestia aryabhattai*, *Enterobacter chuandaensis*, *Burkholderia seminalis*, *Enterobacter cloacae*, *Burkholderia multivorans*, *Bacillus xiamenensis*, and *Chromobacterium violaceum*. Three fungal endophyte isolates were recovered from [n] surface-sterilized root samples on PDA, and no growth was observed from the final rinse water, which confirmed effective surface sterilization. ITS sequence analysis showed that isolates EF1 and EF2 were most closely related to *Trichoderma yunnanense*, and isolate EF3 to *Trichoderma simmonsii.*

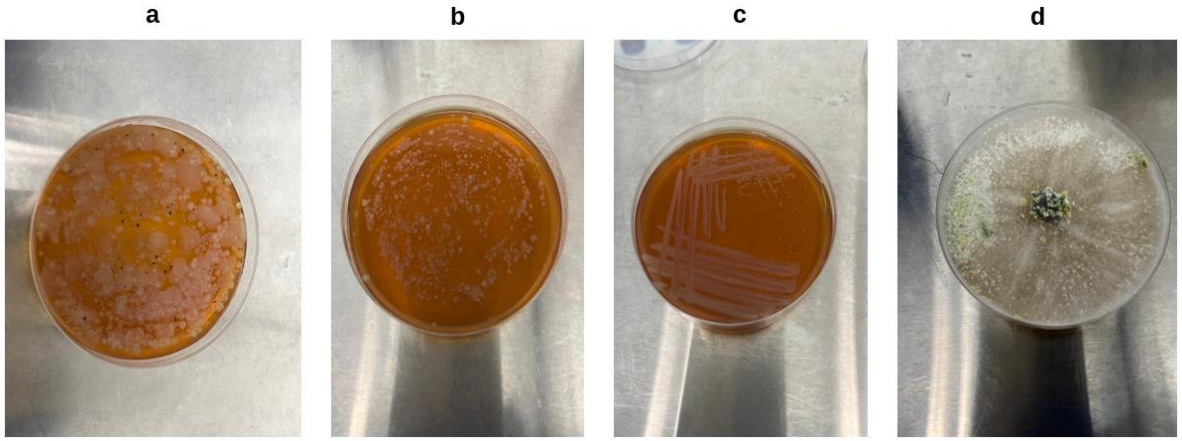


Fig. 1 Representative photographs from the bacterial isolation workflow. (a) Dense initial bacterial growth on LB agar after 24 h incubation of the root suspension. (b) Colony separation following secondary serial dilution (10^{-3}). (c) Purified bacterial colonies obtained by quadrant streaking. (d) Representative fungal endophyte culture. Images shown are representative of the five root samples and eleven purified isolates processed using an identical protocol

3.2 Molecular identification and phylogenetic analysis

The 14 isolates were identified by BLASTn analysis against the NCBI database, using the near full-length 16S rRNA gene sequences (1,413-1,492 bp) of the 11 bacterial isolates and the ITS sequences (608-630 bp) of the three fungal endophytes. The closest matches, percentage identities, and accession numbers are summarized in Table 1. All isolates matched reference sequences at 99.08-100.00% identity, which allowed each to be assigned to a genus with high confidence.

Table 1 Closest NCBI BLASTn matches for the 16S rRNA gene sequences of the bacterial isolates (Bacteria 1–11) and the ITS sequences of the fungal endophytes (Fungi G(G), G(B) and K).

Sample	Top NCBI BLAST match	Identity (%)	Accession
Bacteria 1	*Priestia megaterium*	99.93	NR_117473.1
Bacteria 2	*Priestia megaterium*	99.93	NR_117473.1
Bacteria 3	*Bacillus cereus* ATCC 14579	100.00	NR_074540.1
Bacteria 4	*Bacillus cereus* ATCC 14579	100.00	NR_074540.1

Sample	Top NCBI BLAST match	Identity (%)	Accession
Bacteria 5	*Priestia aryabhattai* B8W22	99.93	NR_115953.1
Bacteria 6	*Enterobacter chuandaensis*	99.46	NR_180237.1
Bacteria 7	*Burkholderia seminalis*	99.73	NR_042635.1
Bacteria 8	*Enterobacter cloacae*	99.08	NR_117679.1
Bacteria 9	*Burkholderia multivorans*	99.80	NR_029358.1
Bacteria 10	*Bacillus xiamenensis*	99.87	NR_148244.1
Bacteria 11	*Chromobacterium violaceum*	100.00	NR_113595.1
Fungi G(G)	*Trichoderma yunnanense*	99.15	NR_134419.1
Fungi G(B)	*Trichoderma yunnanense*	99.32	NR_134419.1
Fungi K	*Trichoderma simmonsii*	99.51	NR_137297.1

Six of the 11 bacterial isolates belonged to the family Bacillaceae *sensu lato*. Bacteria 1 and Bacteria 2 both matched *Priestia megaterium* (NR_117473.1) at 99.93% identity, and Bacteria 5 matched *Priestia aryabhattai* B8W22 (NR_115953.1) at 99.93%. The genus *Priestia* was proposed to accommodate the *Bacillus megaterium* group, so these isolates were formerly classified under *Bacillus*. Bacteria 3 and Bacteria 4 were identical (100.00%) to *Bacillus cereus* ATCC 14579 (NR_074540.1), and Bacteria 10 matched *Bacillus xiamenensis* (NR_148244.1) at 99.87%. Despite these high identities, the next-best matches were often other named species of the same group. Bacteria 1 also showed 99.87% identity to *P. aryabhattai*; Bacteria 3 and 4 showed 99.80–99.93% identity to *B. proteolyticus*, *B. albus* and *B. paranthracis* (and, for Bacteria 4, *B. sanguinis* and *B. wiedmannii*); and Bacteria 10 showed 99.93% identity to *B. altitudinis* and 99.87% to *B. stratosphericus*. The species-level assignments for these isolates are therefore putative.

The remaining five bacterial isolates were Gram-negative Proteobacteria. Bacteria 6 and Bacteria 8 were assigned to the genus *Enterobacter*, matching *E. chuandaensis* (NR_180237.1) at 99.46% and *E. cloacae* (NR_117679.1) at 99.08%, respectively. These were the lowest identities in the bacterial dataset, and both isolates also had

near-equal matches to other Enterobacteriaceae (Bacteria 6: *E. cancerogenus* and *Klebsiella pasteurii*, 99.32%; Bacteria 8: *Kosakonia oryzendophytica*, 99.93% over 97% query coverage). Bacteria 7 and Bacteria 9 matched *Burkholderia seminalis* (NR_042635.1; 99.73%) and *Burkholderia multivorans* (NR_029358.1; 99.80%), both members of the *Burkholderia cepacia* complex (Bcc), whose species are very similar in 16S rRNA gene sequence; Bacteria 7, for example, also shared 99.73% identity with *B. paludis*, *B. anthina* and *B. contaminans*. Bacteria 11 was identical (100.00%) to *Chromobacterium violaceum* (NR_113595.1), whereas other species of the genus showed lower identities (98.30–98.57%), making this the least ambiguous assignment.

The ITS sequences of the three fungal endophytes matched members of the genus *Trichoderma*. Fungi G(G) and Fungi G(B) were most similar to *Trichoderma yunnanense* (NR_134419.1), at 99.15% and 99.32% identity, respectively, and Fungi K was most similar to *Trichoderma simmonsii* (NR_137297.1) at 99.51%. Because two of the three identities were below 99.5% and the ITS region has limited power to separate closely related *Trichoderma* species, the fungal identifications are provisional at species level.

The phylogenetic relationships of the bacterial isolates were analyzed in MEGA X and are shown in Fig. 2. The tree contains the 11 bacterial 16S rRNA gene sequences, labelled by their closest reference strain, and resolves two major lineages: a Gram-positive Bacillaceae lineage (*Priestia* and *Bacillus*; bootstrap support 100%) and a Gram-negative Proteobacteria lineage (*Enterobacter*, *Chromobacterium* and *Burkholderia*). Within the Bacillaceae lineage, the isolates matching *P. megaterium* and *P. aryabhattai* formed a single cluster (bootstrap 95–99%), the two *B. cereus* isolates formed a separate cluster (99%), and *B. xiamenensis* branched basal to both. The grouping of the *Priestia* and *B. cereus* clusters was only weakly supported (53%), so the relationship between them is not resolved. Branch lengths among the *Priestia* and *B. cereus* sequences were close to zero, reflecting their near-identical 16S rRNA genes. Within the Proteobacteria lineage, *E. chuandaensis* and *E. cloacae* formed a strongly supported *Enterobacter* cluster (100%), *C. violaceum* grouped with the two *Burkholderia* species (99%), and *B. seminalis* and *B. multivorans* were sister taxa (99%).

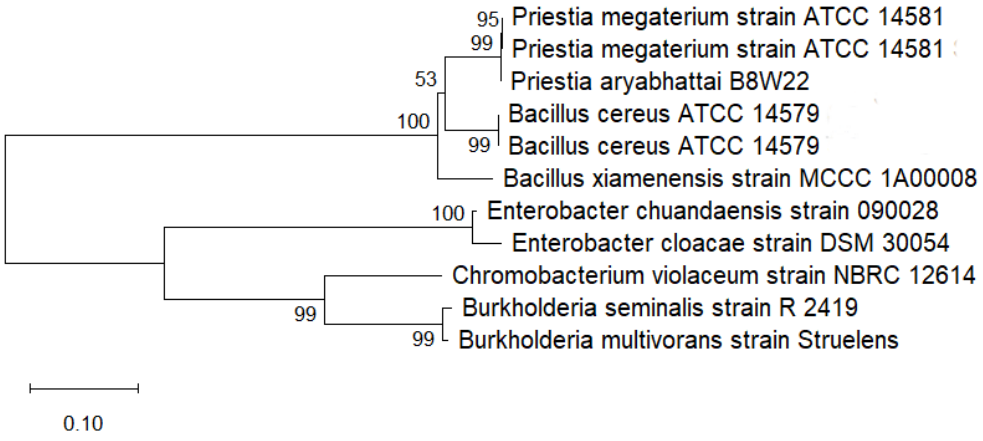


Fig. 2 Phylogenetic tree of the 16S rRNA gene sequences of the 11 bacterial isolates, labelled by their closest NCBI reference strain, constructed in MEGA X by the Neighbor-Joining method. Numbers at the nodes are bootstrap values (%). The scale bar represents 0.10 substitutions per nucleotide site

Overall, the phylogenetic placement of each bacterial isolate was congruent with its BLASTn assignment. Every isolate clustered with its top-matching reference strain, isolates assigned to the same species (Bacteria 1 and 2; Bacteria 3 and 4) grouped together, and no isolate fell outside the genus indicated by BLASTn. The tree is based on the 16S rRNA gene of the bacterial isolates only; the identification of the fungal endophytes rests on BLASTn similarity of the ITS region.

3.3 Nitrogen-fixation screening

Presumptive nitrogen-fixing ability was screened on nitrogen-free Jensen's agar. Because this medium contains no added nitrogen source, growth on it is taken as a presumptive indicator of diazotrophic potential (the ability to fix atmospheric N_2), not as proof of it. All 11 bacterial isolates grew on the medium (Table 2). Representative growth is shown in Fig. 3: *Priestia megaterium* (Bacteria 1) formed strong, mucoid colonies (Fig. 3a), *Bacillus cereus* (Bacteria 4) grew strongly (Fig. 3b), and *Chromobacterium violaceum* (Bacteria 11) formed pigmented colonies (Fig. 3c).

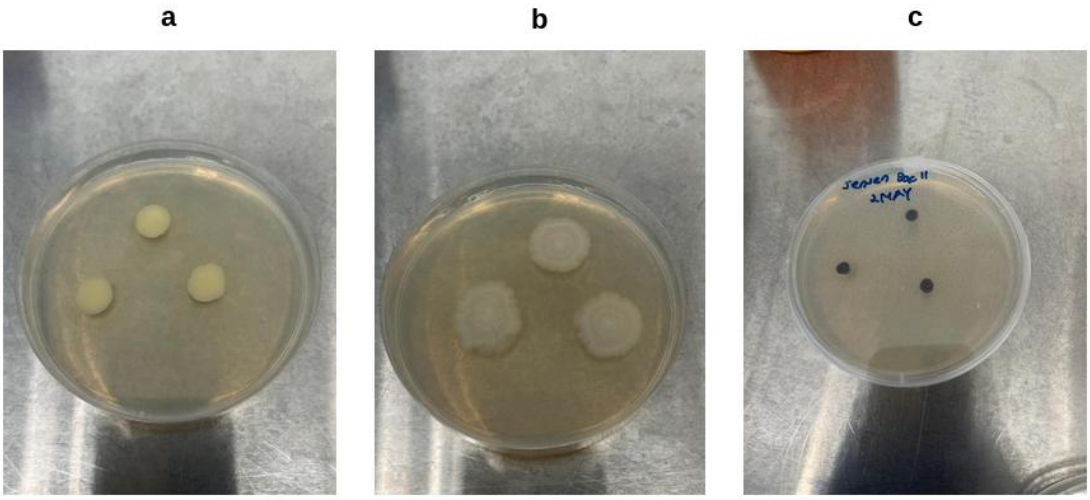


Fig. 3 Qualitative nitrogen-fixation screening on Jensen's nitrogen-free agar after incubation. (a) Isolated Bacteria 1 (*Priestia megaterium*) showing strong/mucoid colony growth. (b) Isolated Bacteria 4 (*Bacillus cereus*) showing strong growth. (c) Isolated Bacteria 11 (*Chromobacterium violaceum*) showing pigmented colony growth

Growth on nitrogen-free medium is not unique to diazotrophs, as the result for Bacteria 11 shows. This isolate was identified as *C. violaceum* by 16S rRNA gene sequencing (100% identity), and this species is generally regarded as non-diazotrophic (*13*), so its growth most likely reflects trace or carried-over nitrogen. Growth alone therefore cannot separate true nitrogen fixers from isolates that persist on residual nitrogen, and the diazotrophic activity of the other isolates needs confirmation by the acetylene reduction assay (ARA) or *nifH* gene amplification.

Table 2 Growth and qualitative nitrogen-fixation capability of bacterial and fungal isolates on nitrogen-free Jensen's medium.

Isolate	Growth	Nitrogen Fixation
Priestia megaterium (Bacteria 1)	Positive	Yes
Priestia megaterium (Bacteria 2)	Positive	Yes

Isolate	Growth	Nitrogen Fixation
Bacillus cereus (Bacteria 3)	Positive	Variable/Weak
Bacillus cereus (Bacteria 4)	Positive	Variable/Weak
Priestia aryabhattai (Bacteria 5)	Positive	Yes
Enterobacter chuandaensis (Bacteria 6)	Positive	Yes
Burkholderia seminalis (Bacteria 7)	Positive	Yes
Enterobacter cloacae (Bacteria 8)	Positive	Yes
Burkholderia multivorans (Bacteria 9)	Positive	Yes
Bacillus xiamenensis (Bacteria 10)	Positive	Yes
Chromobacterium violaceum (Bacteria 11)	Positive	No (lacks nitrogenase genes)
Trichoderma yunnanense (Fungi G(G))	Positive	No (fungi lack nitrogenase)
Trichoderma yunnanense (Fungi G(B))	Positive	No (fungi lack nitrogenase)
Trichoderma simmonsii (Fungi K)	Positive	No (fungi lack nitrogenase)

3.4 Phosphate solubilization assay

Phosphate-solubilizing ability was assessed qualitatively on Pikovskaya's (PVK) agar containing tricalcium phosphate, $Ca_3(PO_4)_2$ (Fig. 4; Table 3). Of the 14 isolates tested (11 bacteria and three fungi), only *Burkholderia seminalis* (Bacteria 7) and *Burkholderia multivorans* (Bacteria 9) formed clear halos around their colonies. Bacteria 7 showed the highest activity, with a mean colony diameter of 15.67 mm, a

mean halo of 22.67 mm, and an SI of 2.45. Bacteria 9 also showed clear solubilization, with a mean colony diameter of 10.33 mm, a mean halo of 12.00 mm, and an SI of 2.16. Bacteria 1-6, 10, and 11 and all three fungal isolates (G(G), G(B), and K) grew on PVK agar but formed no visible halo (SI = 1.00), so no phosphate solubilization was detected under these plate conditions. For Bacteria 8 (*Enterobacter cloacae*), the halo could not be measured because the agar broke during incubation (Table 3).

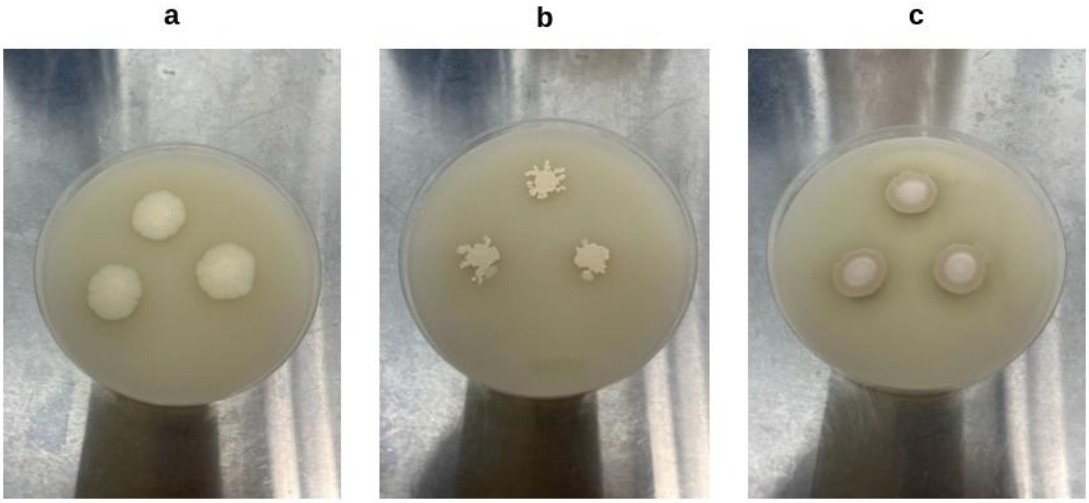


Fig. 4 Inorganic phosphate solubilization on Pikovskaya's (PVK) agar after incubation. (a) Bacteria 1 showing growth with no visible halo zone. (b) Bacteria 4 showing growth with no visible halo zone. (c) Bacteria 7 (*Burkholderia seminalis*) showing a clear solubilization halo around the colony

Table 3 Solubilization index (SI) of bacterial and fungal isolates on Pikovskaya's (PVK) agar after incubation.

Isolate	Avg. colony diameter (mm)	Avg. halo diameter (mm)	SI
Priestia megaterium (Bacteria 1)	Growth	No halo zone	1.00
Priestia megaterium (Bacteria 2)	Growth	No halo zone	1.00
Bacillus cereus (Bacteria 3)	Growth	No halo zone	1.00

Isolate	Avg. colony diameter (mm)	Avg. halo diameter (mm)	SI
Bacillus cereus (Bacteria 4)	Growth	No halo zone	1.00
Priestia aryabhattai (Bacteria 5)	Growth	No halo zone	1.00
Enterobacter chuandaensis (Bacteria 6)	Growth	No halo zone	1.00
Burkholderia seminalis (Bacteria 7)	15.67	22.67	2.447
Enterobacter cloacae (Bacteria 8)	Agar break	Agar break	–
Burkholderia multivorans (Bacteria 9)	10.33	12.00	2.162
Bacillus xiamenensis (Bacteria 10)	Growth	No halo zone	1.00
Chromobacterium violaceum (Bacteria 11)	Growth	No halo zone	1.00
Trichoderma yunnanense (Fungi G(G))	Growth	No halo zone	1.00
Trichoderma yunnanense (Fungi G(B))	Growth	No halo zone	1.00
Trichoderma simmonsii (Fungi K)	Growth	No halo zone	1.00

3.5 Potassium solubilization assay

Potassium-solubilizing ability was assessed qualitatively on Aleksandrov agar containing insoluble potassium aluminosilicate (potassium feldspar) as the K source (Fig. 5; Table 4). Solubilization was indicated by a clear halo around the colony. Of the 14 isolates tested (11 bacteria and three fungi), only *Burkholderia seminalis* (Bacteria 7) and *Burkholderia multivorans* (Bacteria 9) formed halos. Bacteria 9 had the highest

SI (2.83), with a mean colony diameter of 6.00 mm and a mean halo of 11.00 mm. Bacteria 7 had a mean colony diameter of 8.00 mm, a mean halo of 11.00 mm, and an SI of 2.38. All other isolates (Bacteria 1-6, 8, 10, and 11, and fungal isolates G(G), G(B), and K) grew on the medium but formed no halo (SI = 1.00). The two isolates that solubilized potassium were also the only ones that solubilized tricalcium phosphate, which suggests that they may combine both traits.

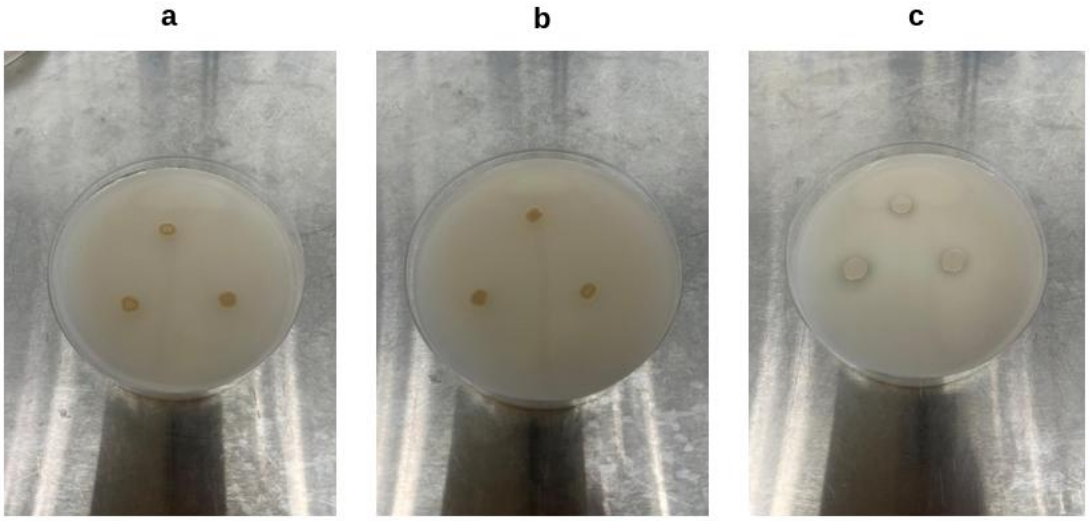


Fig. 5 Potassium solubilization on Aleksandrov agar after incubation. (a) Bacteria 1 showing growth with no visible halo zone. (b) Bacteria 5 showing growth with no visible halo zone. (c) Bacteria 7 (*Burkholderia seminalis*) showing a clear solubilization halo around the colony

Table 4 Solubilization index (SI) of bacterial and fungal isolates on Aleksandrov agar after incubation.

Isolate	Avg. colony diameter (mm)	Avg. halo diameter (mm)	SI
Priestia megaterium (Bacteria 1)	Growth	No halo zone	1.00
Priestia megaterium (Bacteria 2)	Growth	No halo zone	1.00
Bacillus cereus (Bacteria 3)	Growth	No halo zone	1.00

Isolate	Avg. colony diameter (mm)	Avg. halo diameter (mm)	SI
Bacillus cereus (Bacteria 4)	Growth	No halo zone	1.00
Priestia aryabhattai (Bacteria 5)	Growth	No halo zone	1.00
Enterobacter chuandaensis (Bacteria 6)	Growth	No halo zone	1.00
Burkholderia seminalis (Bacteria 7)	8.00	11.00	2.375
Enterobacter cloacae (Bacteria 8)	Growth	No halo zone	1.00
Burkholderia multivorans (Bacteria 9)	6.00	11.00	2.833
Bacillus xiamenensis (Bacteria 10)	Growth	No halo zone	1.00
Chromobacterium violaceum (Bacteria 11)	Growth	No halo zone	1.00
Trichoderma yunnanense (Fungi G(G))	Growth	No halo zone	1.00
Trichoderma yunnanense (Fungi G(B))	Growth	No halo zone	1.00
Trichoderma simmonsii (Fungi K)	Growth	No halo zone	1.00

3.6 Zinc solubilization assay

Zinc-solubilizing ability was assessed qualitatively on zinc-amended agar containing insoluble zinc oxide (ZnO) (Fig. 6; Table 5). Of the 14 isolates tested, five bacteria formed clear halos around their colonies: *Bacillus cereus* (Bacteria 3 and 4), *Enterobacter chuandaensis* (Bacteria 6), *Enterobacter cloacae* (Bacteria 8), and *Bacillus xiamenensis* (Bacteria 10). Bacteria 4 had the highest solubilization index (SI

= 2.57; colony 10.00 mm, halo 15.67 mm), followed by Bacteria 6 (2.44), Bacteria 8 (2.41), Bacteria 3 (2.35), and Bacteria 10 (2.09). *Priestia megaterium* (Bacteria 1 and 2) and *Priestia aryabhattai* (Bacteria 5) did not grow on the medium. Bacteria 7, 9, and 11 and all three fungal isolates (G(G), G(B), and K) grew but formed no halo (SI = 1.00). The zinc-solubilizing isolates were therefore different from those that solubilized phosphate and potassium (Sections 3.4 and 3.5), which were the two *Burkholderia* isolates (Bacteria 7 and 9).

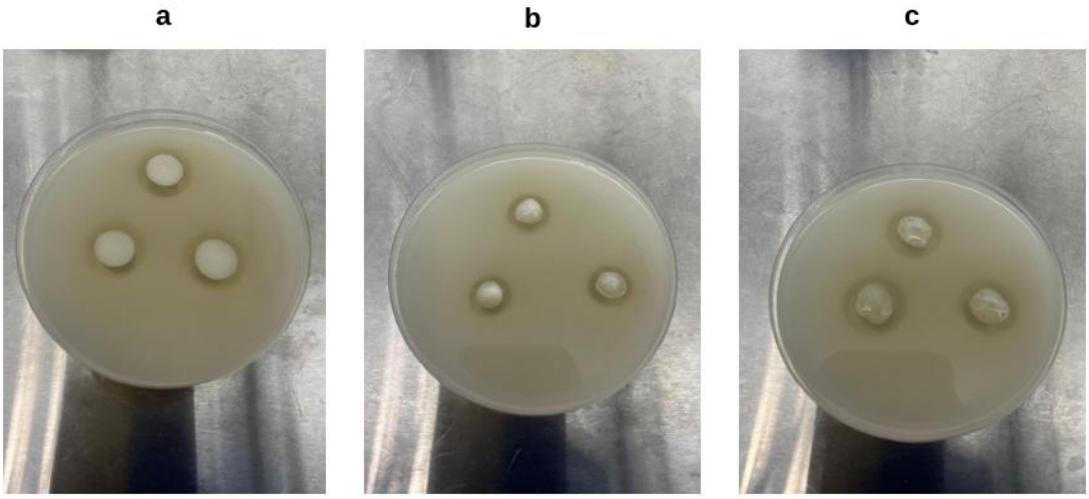


Fig. 6 Zinc solubilization on zinc-amended agar after incubation. (a) Bacteria 3 (*Bacillus cereus*) showing a clear solubilization halo. (b) Bacteria 6 (*Enterobacter chuandaensis*) showing a clear solubilization halo. (c) Bacteria 8 (*Enterobacter cloacae*) showing a clear solubilization halo

Table 5 Solubilization index (SI) of bacterial and fungal isolates on zinc solubilizing agar after incubation.

Isolate	Avg. colony diameter (mm)	Avg. halo diameter (mm)	SI
Priestia megaterium (Bacteria 1)	No growth	No growth	–
Priestia megaterium (Bacteria 2)	No growth	No growth	–

Isolate	Avg. colony diameter (mm)	Avg. halo diameter (mm)	SI
Bacillus cereus (Bacteria 3)	12.33	16.67	2.352
Bacillus cereus (Bacteria 4)	10.00	15.67	2.567
Priestia aryabhattai (Bacteria 5)	No growth	No growth	–
Enterobacter chuandaensis (Bacteria 6)	9.00	13.00	2.444
Burkholderia seminalis (Bacteria 7)	Growth	No halo zone	1.00
Enterobacter cloacae (Bacteria 8)	12.33	17.33	2.406
Burkholderia multivorans (Bacteria 9)	Growth	No halo zone	1.00
Bacillus xiamenensis (Bacteria 10)	7.33	8.00	2.091
Chromobacterium violaceum (Bacteria 11)	Growth	No halo zone	1.00
Trichoderma yunnanense (Fungi G(G))	Growth	–	–
Trichoderma yunnanense (Fungi G(B))	Growth	–	–
Trichoderma simmonsii (Fungi K)	Growth	–	–

3.7 Ammonia production assay

Ammonia production was assessed qualitatively in peptone water using Nessler's reagent (Fig. 7; Table 6). The uninoculated control tube stayed pale straw-yellow, with no precipitate or color change (Fig. 7a), which shows that the medium alone gave no

false-positive reaction. *Priestia megaterium* (Bacteria 1) produced a clear shift to yellow, with turbidity from the cell suspension (Fig. 7b), and *Enterobacter cloacae* (Bacteria 8) produced a more intense yellow-to-light-orange color (Fig. 7c). All 14 isolates (11 bacteria and three fungi) were scored positive (+) for ammonia production (Table 6), so this assay did not discriminate between isolates. This suggests that ammonia release from peptone is a common trait among the isolates, but the test is qualitative and does not show how much ammonia each isolate produced.

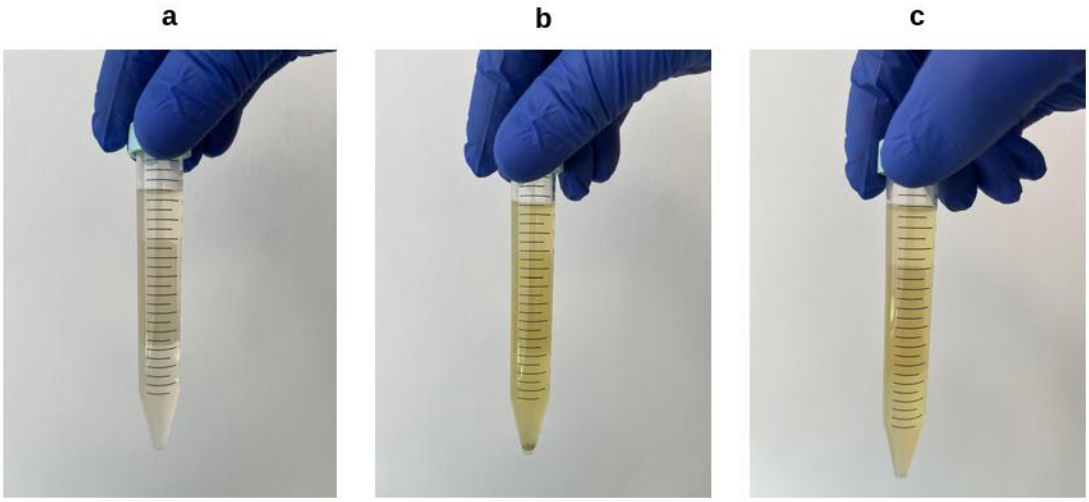


Fig. 7 Ammonia production assay in peptone water following the addition of Nessler's reagent. (a) Uninoculated control showing a pale straw-yellow appearance with no reaction. (b) Culture inoculated with Bacteria 1 (*Priestia megaterium*) showing a shift to yellow with visible turbidity/pellet formation. (c) Culture inoculated with Bacteria 8 (*Enterobacter cloacae*) showing a strong yellow-to-light-orange color transition

Table 6 Ammonia production by bacterial and fungal isolates, detected by adding Nessler's reagent to liquid peptone cultures following incubation. Development of a yellow, orange, or brownish color change indicates a positive (+) result for ammonia (NH_3) synthesis.

Isolate	Initial culture colour	Post-Nessler's reagent colour	Ammonia production
Control (Uninoculated)	Pale yellow	Pale straw / clear yellow	Negative
Priestia megaterium (Bacteria 1)	Pale yellow	Yellow with noticeable bottom pellet	Positive
Priestia megaterium (Bacteria 2)	Pale yellow	Light yellow with slight bottom settlement	Positive
Bacillus cereus (Bacteria 3)	Pale yellow	Yellow with distinct yellow-green bottom pellet	Positive
Bacillus cereus (Bacteria 4)	Pale yellow	Light yellow-green with bottom settlement	Positive
Priestia aryabhattai (Bacteria 5)	Pale yellow	Yellow with dark green-brown bottom pellet	Positive
Enterobacter chuandaensis (Bacteria 6)	Pale yellow	Light yellow with greenish bottom pellet	Positive
Burkholderia seminalis (Bacteria 7)	Pale yellow	Yellow with dense yellow-green bottom pellet	Positive
Enterobacter cloacae (Bacteria 8)	Pale yellow	Yellow to light orange with slight turbidity	Positive

Isolate	Initial culture colour	Post-Nessler's reagent colour	Ammonia production
Burkholderia multivorans (Bacteria 9)	Pale yellow	Pale yellow with distinct yellow bottom pellet	Positive
Bacillus xiamenensis (Bacteria 10)	Pale yellow	Light yellow with distinct yellow bottom pellet	Positive
Chromobacterium violaceum (Bacteria 11)	Pale yellow	Light yellow with pale bottom settlement	Positive
Trichoderma yunnanense (Fungi G(G))	Pale yellow	Deep yellow	Positive
Trichoderma yunnanense (Fungi G(B))	Pale yellow	Deep yellow	Positive
Trichoderma simmonsii (Fungi K)	Pale yellow	Deep yellow	Positive

3.8 Selection of candidate strains for a multi-trait bioinoculant consortium

No single isolate showed all four screened traits (Sections 3.3-3.6), so candidate strains for a multi-trait consortium were selected using two criteria: (i) coverage of presumptive nitrogen fixation and of phosphate, potassium, and zinc solubilization, and (ii) redundancy, so that a trait was supported by more than one isolate where the screening data allowed. Ammonia production was positive for all isolates (Section 3.7) and therefore did not contribute to selection. Growth on nitrogen-free Jensen's medium is a presumptive indicator of diazotrophic potential only and was prioritized over SI when choosing among the zinc solubilizers. *Bacillus cereus* (Bacteria 3, SI = 2.35; Bacteria 4, SI = 2.57) solubilized zinc but showed weak or variable growth on

nitrogen-free medium (Table 2). *Enterobacter chuandaensis* (Bacteria 6, SI = 2.44) and *Enterobacter cloacae* (Bacteria 8, SI = 2.41) combined zinc solubilization with growth on nitrogen-free medium and were therefore selected as a redundant zinc-solubilizing pair. *Burkholderia seminalis* (Bacteria 7) solubilized both phosphate (SI = 2.45) and potassium (SI = 2.38) and grew on nitrogen-free medium and was selected to cover these two traits. *Burkholderia multivorans* (Bacteria 9) showed the same combination of traits (phosphate SI = 2.16; potassium SI = 2.83) but was excluded [because it belongs to the *B. cepacia* complex (Bcc), which includes opportunistic pathogens]. As a result, zinc solubilization is supported by two strains, whereas phosphate and potassium solubilization rest on a single strain (Table 7). *Priestia megaterium* and *P. aryabhattai* (Bacteria 1, 2, and 5) did not grow on the zinc-amended medium (Section 3.6), so they could not be evaluated for zinc solubilization and were not considered further.

Table 7 Candidate isolates for a multi-trait bioinoculant consortium, selected for trait coverage and growth on nitrogen-free medium.

Isolate	Growth on N-free medium	P (SI)	K (SI)	Zn (SI)	Role in consortium
Enterobacter chuandaensis (Bacteria 6)	+	1.00	1.00	2.44	Zinc solubilizer
Burkholderia seminalis (Bacteria 7)	+	2.45	2.38	1.00	Phosphate and potassium solubilizer (only strain for these traits)
Enterobacter cloacae (Bacteria 8)	+	n.d.[a]	1.00	2.41	Zinc solubilizer (redundancy)

[a] n.d. = not determined; the phosphate solubilization halo could not be measured due to agar breakage during incubation (Section 3.4).

The resulting three-strain combination (Table 7) has two zinc-solubilizing isolates (Bacteria 6 and 8) and a single strain (Bacteria 7) for phosphate and potassium solubilization. All three strains grew on nitrogen-free Jensen's medium (Table 2), and all were positive for ammonia production (Table 6), as were all other isolates. On this basis, *E. chuandaensis* (Bacteria 6), *B. seminalis* (Bacteria 7), and *E. cloacae* (Bacteria 8) are proposed as candidates for further evaluation in a multi-trait consortium. This selection is based on qualitative plate screens only and is intended to identify strains for further testing, not to recommend them for field use. Several of the selected species, including *E. cloacae* and members of the *B. cepacia* complex, include opportunistic human pathogens, so biosafety must be assessed [e.g., by genome-based screening for virulence genes and by strain-level testing] before any application. In addition, compatibility among the three strains [was tested by cross-streaking and no inhibition was observed / was not tested and remains to be determined], and nitrogen fixation, nutrient solubilization, and plant growth promotion need to be confirmed by quantitative assays (e.g., ARA or *nifH* amplification, and soluble P, K, and Zn measurements in liquid culture) and by plant trials.

4. Discussion

Culture-based isolation from the rhizosphere and roots of *Barleria lupulina* gave 11 bacterial isolates representing nine species in five genera (*Priestia*, *Bacillus*, *Enterobacter*, *Burkholderia*, and *Chromobacterium*), along with three fungal endophyte isolates from two *Trichoderma* species. Medicinal and herbal plants are often reported to support rhizosphere communities that are richer and functionally more varied than those of non-medicinal crops, possibly because plant secondary metabolites and root exudates select for microbes (*14*). Our results fit this idea, but we did not compare *B. lupulina* with other plants or profile the whole community, so they cannot test it. Even so, the collection suggests that herbal rhizospheres are worth exploring as a source of plant growth-promoting candidates.

Growth on nitrogen-free Jensen medium and ammonia production were widespread among the isolates, in line with earlier reports that diazotrophic and ammonifying activity is common among rhizosphere heterotrophs (*15, 16*). One result show why the nitrogen screen needs caution. *Chromobacterium violaceum* (Bacteria 11) grew on the

nitrogen-free medium even though this species is not generally regarded as a diazotroph and is not known to carry the *nif* genes needed for nitrogenase. Its growth most likely reflects survival on trace or carried-over nitrogen, so visible growth alone cannot prove nitrogen fixation. The isolates that grew should therefore be treated as presumptive diazotrophs until they are confirmed by *nifH* PCR or the acetylene reduction assay (*17, 18*).

Phosphate and potassium solubilization were much less common. Only Bacteria 7 (*Burkholderia seminalis*) and Bacteria 9 (*Burkholderia multivorans*) formed clear halos on both Pikovskaya and Aleksandrov agar, with solubilization indices above 2.1. The fact that the same two isolates showed both traits suggests that they may mobilize more than one mineral nutrient, possibly through organic acid production, as described for other multifunctional plant growth-promoting rhizobacteria, although we did not test the mechanism (*19*). Zinc solubilization followed a different pattern. It occurred in *B. cereus* (Bacteria 3 and 4), *E. chuandaensis* (Bacteria 6), *E. cloacae* (Bacteria 8), and *B. xiamenensis* (Bacteria 10), but not in the *Burkholderia* isolates. This suggests that phosphate/potassium and zinc solubilization are not usually inherited together, which agrees with reports that zinc solubilization is often strain- and mineral-specific. Because no single isolate showed all four traits, a multi-strain consortium looks more promising than relying on one best isolate, an approach reported to widen the range of nutrients available to the host plant (*20*).

B. multivorans (Bacteria 9) exhibited the same two traits but was excluded from the consortium. Because both *Burkholderia* isolates belong to the *Burkholderia cepacia* complex (BCC), only one was retained to minimize potential biosafety liabilities, and B. seminalis was prioritized due to its higher phosphate solubilization index (SI). Furthermore, *B. multivorans* is more strongly documented as a severe opportunistic human pathogen, particularly in cystic fibrosis-associated respiratory infections (*21, 22*). Consequently, *B. seminalis* is maintained solely as a candidate pending rigorous biosafety and virulence evaluation. All three selected strains grew on nitrogen-free medium and produced ammonia, collectively covering the four screened functional traits, though the nitrogen-fixation phenotype remains presumptive. While zinc solubilization is supported by two distinct strains, phosphate and potassium solubilization rely entirely on a single isolate, creating potential vulnerability should that strain fail to colonize or persist under field conditions. Incorporating an additional

phosphate- and potassium-solubilizing isolate would introduce valuable functional redundancy, provided the candidate passes thorough biosafety clearance (*23*). At this stage, these isolates represent candidate strains for controlled downstream evaluation rather than validated formulations for open environmental release.

Plate-based halo assays are a cheap and practical first screen, but they have limits. Solubilization indices calculated from halo measurements depend on agar diffusion, incubation time, and inoculum density, and they do not necessarily reflect nutrient release in soil. The agar breakage that prevented measurement of Bacteria 8 on Pikovskaya agar is another practical drawback. Quantitative assays (for example, molybdenum-blue measurement of phosphate) and greenhouse or field trials are needed to confirm the agronomic relevance of these traits before any strain is taken toward formulation.

All bacterial isolates matched reference sequences at 99.08-100.00% identity (Table 1), and the MEGA X tree placed each isolate with its closest reference strain, mostly with high bootstrap support (95-100%; Fig. 2). This places every isolate confidently at genus level. The tree separated the Gram-positive Bacillaceae (*Priestia* and *Bacillus*) from the Gram-negative Proteobacteria (*Enterobacter*, *Burkholderia*, and *Chromobacterium*), and this split matched the screening results: phosphate and potassium solubilization occurred only in the two *Burkholderia* isolates, which were sister taxa in the tree, while zinc solubilization occurred in *Bacillus* and *Enterobacter* isolates. Isolates of the same species also behaved alike. Both *B. cereus* isolates solubilized zinc, and both *P. megaterium* isolates showed no phosphate or potassium solubilization and did not grow on the zinc medium. In this collection, then, the traits seem to follow taxonomy, although strain-level variation cannot be excluded with so few isolates per species.

The limits of 16S rRNA gene sequencing still need to be kept in view. Top BLAST hits shared more than 99% identity across several named species within the *Priestia megaterium*/*aryabhattai* group, the *Bacillus cereus* sensu lato group, the *Bacillus pumilus* group (where Bacteria 10 matched *B. altitudinis* at a higher identity than *B. xiamenensis*), and the *Burkholderia cepacia* complex. The *B. cereus* group contains species that are difficult to tell apart genetically (*24*). The two *Enterobacter* isolates had the lowest identities (99.46% and 99.08%), and Bacteria 8 matched *Kosakonia*

oryzendophytica more closely than any *Enterobacter* reference, so both may belong to related species within the *E. cloacae* complex and are best regarded as *Enterobacter* sp. until confirmed. The low bootstrap support (53%) for the grouping of *Priestia* and *B. cereus* also shows that a single-gene tree cannot resolve all deeper relationships. Sequence similarity alone should not be used to assign species, so the species names reported here are putative (*25*). Multilocus sequence analysis (for example *gyrB*, *rpoB*, or *recA*) or whole-genome sequencing is recommended to confirm them.

The three fungal endophytes were assigned to *Trichoderma*, with Fungi G(G) and G(B) matching *T. yunnanense* and Fungi K matching *T. simmonsii* at 99.15-99.51% identity. ITS is the recommended primary barcode for fungi, but it does not reliably separate species within *Trichoderma*, including the *T. harzianum* species complex to which *T. simmonsii* belongs (*26, 27*). Reliable species identification generally requires additional loci such as *tef1* and *rpb2*, and we did not build a fungal phylogeny. The fungal species names are therefore provisional.

The identification results also raise a biosafety question (*28*). The *Bacillus cereus* group, the *Burkholderia cepacia* complex, the *Enterobacter cloacae* complex, and *Chromobacterium violaceum* all include species that can act as opportunistic pathogens [19, 25]. Notably, all three strains proposed for the consortium in Section 3.8 belong to *Enterobacter* (Bacteria 6 and 8) or the *Burkholderia cepacia* complex (Bacteria 7). Before any isolate from this study is considered for field use, multilocus or whole-genome sequencing, together with pathogenicity and toxin-gene screening, is needed to confirm species identity and rule out safety concerns (*29*). If these checks are unfavorable, alternative isolates from this collection would have to be considered.

5. Conclusion

This study shows that the rhizosphere and roots of the herbal plant *Barleria lupulina* harbor a diverse set of culturable bacteria and fungal endophytes with plant growth-promoting potential. We obtained 11 bacterial isolates representing nine species, together with three *Trichoderma* isolates (*T. yunnanense* and *T. simmonsii*), identified by 16S rRNA and ITS sequencing. BLASTn identities ranged from 99.08% to 100.00%, and phylogenetic analysis placed each bacterial isolate with its closest

reference strain, although species-level assignments within closely related complexes remain putative.

Growth on nitrogen-free medium and ammonia production were widespread among the isolates, whereas phosphate and potassium solubilization were limited to two *Burkholderia* isolates and zinc solubilization to a separate group of *Bacillus* and *Enterobacter* isolates. No single isolate showed all four traits, which supports a multi-strain consortium approach to bioinoculant development.

Future work should confirm nitrogen fixation at the molecular level, validate nutrient solubilization quantitatively under greenhouse and field conditions, and screen the isolates for biosafety, particularly those belonging to species complexes with known pathogenic members. Only after these steps should the most promising strains be considered for formulation as a multifunctional bioinoculant for sustainable agriculture.

Declarations

Author contributions

M.S.L.C. conducted the experiments, analyzed the data, and drafted the original manuscript. **A.S.A.S**. contributed to the critical review and editing of the manuscript. **N.R.** performed experimental work and participated in manuscript review and editing. **N.M.S.** provided resources, oversaw nursery facility operations, and contributed to project administration. **N.S.R**. conceptualized the study, performed experimental procedures, wrote the initial draft, and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Data availability

The sequence datasets generated and analysed during the current study have been deposited in the NCBI repository under BioProject accession number PRJNA1550021 (accessible at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1550021)

Ethics approval and consent to participate

Not applicable (no human or animal subjects)

Competing interests

The authors declare that they have no competing interests.

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Consent to Publication

Not applicable

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