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🌐 Defying Salinity, Drought, and pH Extremes: A Multifunctional Rhizobacterium, Burkholderia gladioli ST3M-39a, Matches Fertilizer Efficacy in Wheat via Phosphate Solubilization

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Abstract

Global phosphorus scarcity and the environmental impacts of chemical fertilizers necessitate sustainable microbial alternatives for agriculture. We characterized *Burkholderia gladioli* ST3M-39a, a maize rhizosphere isolate, as a multifunctional plant growth-promoting rhizobacterium with exceptional climate resilience. The strain achieved rapid phosphate solubilization ($177.96 \pm 5.26 \mu\text{g/mL}$ within 24 h; molybdenum-antimony assay), zinc solubilization, and ammonia production, EPS production, produced stress-alleviating enzymes (cellulase and protease), and biosurfactant production. Crucially, it maintained robust growth and phosphate-mobilizing capacity under extreme abiotic stresses: pH 4.5–8.5, 7.5% NaCl salinity, and drought-mimicking low water activity (a_w 0.950, 32% sorbitol). The strain was non-hemolytic and non-pathogenic to plants. In wheat trials, ST3M-39a inoculation significantly increased the growth parameters ($p < 0.05$ vs. those of the uninoculated controls), resulting in 85–92% of the biomass stimulation observed with diammonium phosphate (DAP) fertilizer. This multifunctional stress tolerance, coupled with its near-fertilizer efficacy, positioned ST3M-39a as a transformative bioinoculant for degraded soils. Field validation of its agricultural deployment and ecological impact is now pivotal.

Materials

All microbiological growth media and general reagents were procured from HiMedia and Fisher Scientific. Molecular biology-grade reagents, buffers, and PCR master mix were sourced from Amresco and New England Biolabs, respectively, with PCR primers synthesized by Macrogen (South Korea). Laboratory glassware was supplied by Borosil. Microbial characterization employed an Olympus CX21i microscope. DNA extraction and other centrifugation utilized a REMI NEYA 16R centrifuge, PCR amplification was performed on a MiniAmp™ Plus Thermal Cycler (Thermo Fisher Scientific), and spectrophotometric measurements used a Labdex LX111VS instrument.

Isolation and maintenance of organisms

- 1 Organisms were isolated from rhizospheric soil of maize plants collected in Dumre beshi, Bharatpur-29, Chitwan, Nepal (27°48'52"N and 084°28'47"E) as shown in Figure 1. The soil samples (1 g) were homogenized in 10 ml of sterile water, serially diluted, and spread onto yeast mannitol agar. The plates were incubated aerobically at 30°C for 24 h. Morphologically distinct bacterial colonies were subcultured on glucose-yeast extract-peptone (GYP; 1% glucose, 0.5% yeast extract, 0.3% peptone) agar to obtain pure cultures. Isolates were preserved as glycerol stocks at -80°C for long-term storage and maintained on bacterial slants(stored at 4°C) for experimental use (1).

Screening for phosphate solubilization

- 2 For qualitative assay, Isolates were screened for phosphate solubilization on Pikovskaya's agar (PKA) (2) and National Botanical Research Institute's phosphate (NBRIP) media (3). Both media were incubated at 30°C for 72 h under aerobic conditions. Colonies that formed halo zones on both media were identified as phosphate solubilizers. Isolate ST3M-39a, the sole isolate exhibiting phosphate solubilization on both media, was selected for further analysis. The phosphate solubilization index (PSI) was calculated as follows:

$$\text{PSI} = \frac{\text{Colony diameter} + \text{halo region diameter}}{\text{colony diameter}}$$

- 3 For quantitative assay, The strain exhibiting the sole halo zone was selected for quantitative phosphate solubilization analysis. The strain was precultured in nutrient broth (24 h, 30°C, 100 rpm) before the standardized inoculum (OD₆₀₀ 0.6; 2-10% v/v) was aseptically transferred to 30 mL NBRIP medium (pH 6.8) in 250 mL flasks, with uninoculated medium used as a negative control. Cultures were incubated aerobically (30°C, 100 rpm), and 1 mL samples were collected after 24 h for phosphate quantification. After centrifugation (10,000 × g, 5 min, 4°C), the supernatant was analyzed for solubilized phosphate via the molybdenum-antimony colorimetric method (4). The final pH of the medium was also recorded. All the experiments were performed in triplicate.

Identification of organism

- 4 For DNA extraction and sequencing, The bacterial isolate was incubated in nutrient broth at 30°C for 24 h. Genomic DNA was extracted via the Sambrook and Russell method (5), and the 16S rRNA gene was amplified with the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5-TACGGYTACCTTGTTACGACTT-3'). Sequencing was performed by Macrogen (South Korea). The raw sequences were assembled and trimmed in a codon code aligner, and the consensus sequence was subsequently deposited in NCBI GenBank (accession OR143748.1). For phylogenetic analysis, the sequence was compared to the NCBI database via BLASTN (16S rRNA dataset), and 20 closely related sequences were retrieved in FASTA format.
- 5 A maximum likelihood phylogenetic tree was constructed in MEGA12 (6) via the Tamura-Nei model (7), with nodal support assessed via 2,000 bootstrap replicates (8).
- 6 The isolate was identified morphologically and biochemically following *Bergey's Manual of Systematic Bacteriology* (9).

Growth under different abiotic conditions

- 7 Bacterial growth was assessed in nutrient broth under controlled abiotic stresses: pH gradients (4.0-11.5; 0.5-1.0 increments), NaCl concentrations (0-9.0% w/v; pH 6.8), and sorbitol-induced osmotic stress (0-40% w/v; pH 6.8). Water activity (a_w) for sorbitol solutions was calculated following established methods (41). Overnight cultures ($OD_{600} = 0.6$) were aseptically inoculated at 2% (v/v) into fresh NBRIP medium supplemented with test conditions. Cultures underwent aerobic incubation at 30°C with 100 rpm agitation for 48 h. All treatments were replicated in triplicate.

Phosphate solubilization under different abiotic conditions

- 8 Phosphate solubilization was assessed in NBRIP medium under variable stress conditions: pHs (4.5, 7, and 8.5), NaCl (0-7.5% w/v, 2.5% increments; initial pH 6.8), and sorbitol-induced osmotic stress (0-32% w/v, 8% increments; initial pH 6.8). Overnight nutrient broth culture ($OD_{600} = 0.6$) was inoculated at 6% (v/v) into test media. Following 24 h aerobic incubation at 30°C with 100 rpm agitation, solubilized phosphate was quantified using previously described method and final pH recorded. All assays were performed in triplicate.

Other PGPR properties

- 9 Zinc solubilization was assessed by culturing the isolate in mineral salt media supplemented with 0.1% zinc oxide (10). The plates were incubated at 30°C for 7 days, and the zinc solubilization efficiency (ZSE%) was calculated as (clearing zone diameter/colony diameter) $\times$ 100 (11).

- 10 Exopolysaccharide (EPS) production was induced by inoculating 100 mL LB broth with 100 μ L bacterial culture ($OD_{600} = 0.6$) followed by 96 h incubation at 30°C with 100 rpm orbital shaking. Cells were pelleted by centrifugation, and the supernatant treated with two volumes of ice-cold 95% ethanol to precipitate EPS. Precipitates were recovered, dried, and quantified gravimetrically (12, 13)
- 11 For cellulolytic activity, the isolates were grown in basal salt media supplemented with 1% carboxymethyl cellulose (30°C, 48 h). After incubation, the agar plate was stained with 0.1% Congo red solution for 15 min, followed by counterstaining with 1 M NaCl (20 min). Cellulose hydrolysis was identified by clear zones surrounding colonies (14).
- 12 For the protease test, the isolate was cultured on skim milk powder agar (5% skim milk powder) and incubated at 30°C for 48 hours. The clear halo region indicates the production of protease by the isolate (15).
- 13 Ammonia production was qualitatively evaluated via the method by Chen *et al.* (16) by inoculating test strains in peptone broth and incubating them at 30°C for 48 h. Following incubation, 500 μ L of Nessler's reagent was added, with the development of a yellow to brown color indicating presence of ammonia.
- 14 The bacterial isolate was cultivated in a modified minimal salts medium (30°C, 180 rpm, 96 h) composed of (in g L⁻¹): K₂HPO₄, 2.0; KH₂PO₄, 2.0; MgSO₄•7H₂O, 0.2; (NH₄)₂SO₄, 1.0; glucose, 10.0; with trace elements CaCl₂ and FeSO₄•7H₂O each at 0.001 g L⁻¹, pH 7.0 (50). Post-incubation, biomass was removed by centrifugation (10,000 $\times$ g, 15 min) and the cell-free supernatant was aseptically filtered (0.22 μ m). Biosurfactant presence was assessed via a qualitative drop collapse assay, adapted from Bodour and Miller-Maier (17, 18), wherein 2 μ L of paraffin oil was equilibrated overnight at ambient temperature in a 96-well plate lid, followed by the application of 5 μ L of clarified supernatant onto the oil surface; drop spreading or collapse was subsequently documented.

Safety assessment

- 15 A leaf spray assay was performed to assess the potential for plant disease caused by ST3M-39a. Bacterial cells were harvested and resuspended to an OD₆₀₀ of 1.0. This suspension was sprayed onto the leaves of three-week-old bok choy (*Brassica rapa* subsp. *Chinensis*) plants, using a modified version of the method described by Wang *et al* (19). Control plants were sprayed with nutrient broth. The experiment utilized three leaves from different plants per treatment as biological replicates. After 2 weeks, leaf tissues and mesophyll cells were examined for symptoms of damage using light microscopy. Leaf length, breadth, and root length were recorded. The experiment was conducted in four independent biological repeats.

- 16 The hemolytic activity of ST3M-39a was tested by streaking the strain onto blood agar plates supplemented with 5% (v/v) defibrinated sheep blood (20). Plates were incubated at 37°C for 24 hours and examined for zone of hemolysis.

Greenhouse experiment

- 17 A controlled pot trial was conducted using 45 sterile 9-inch diameter pots filled with autoclaved soil. The wheat (*Triticum aestivum*) was sown, and germination occurred one-week post-sowing. The seedlings were thinned to five uniform plantlets per pot and maintained under greenhouse conditions. The treatments included: (A) a negative control (no supplements; 15 pots), (B) a positive control (90 kg/ha diammonium phosphate [DAP]; 15 pots), and (C) test samples (1 mL of phosphate-solubilizing bacterial inoculum, 1×10^9 CFU/mL; 15 pots). Pots received 50 mL autoclaved water weekly.
- 18 At maturity, agronomic parameters shoot length, root length, spike length, chlorophyll content (SPAD meter), dry biomass, yield per pot, and seed number per pot were measured (17).

Statistical analysis

- 19 All quantitative data were analyzed with Origin Pro software and organized using Microsoft Excel. Significance differences among treatment means were determined by one-way analysis of variance (ANOVA). Prior to ANOVA, the assumption of homogeneity of variance was assessed using Levene's test. For datasets meeting this assumption ($p > 0.05$), standard one-way ANOVA was applied. When the assumption was violated ($p < 0.05$), the robust Welch's ANOVA was used. For datasets where the ANOVA or Welch's F-statistics were significant ($p < 0.05$), post hoc tests were used to identify specific differences between group means. Tukey's HSD test was applied following a standard ANOVA, while the Games-Howell procedure was used following a significant Welch's ANOVA, thereby maintaining a family-wise error rate of 5% in pairwise comparisons. For direct pairwise comparisons between treatment and control groups in the pathogenicity assay (leaf length, breadth, and root length), a two-tailed paired sample t-test was employed, testing the null hypothesis of no significant difference. All tests maintained a significance level of $p < 0.05$.

Protocol references

Koh, 2013 (1), Pikovskaya and Pikovskaya, 1948 (2), Nautiyal, 1999 (3), Sambrook and Russell method (5), MEGA12 (6), Tamura-Nei model (7), Felsenstein, 1985 (8), molybdenum-antimony colorimetric method (4), Bergey's Manual of Systematic Bacteriology (9), Bhakat et al., 2021 (10), Gontia-Mishra et al., 2017 (11), Khan and Bano, 2019 (12), Latif et al., 2022 (13), Demissie et al., 2024 (14), Pandey et al., 2022 (15), Chen et al. (16), Bodour and Miller-Maier (17,18), Wang et al (19), Levene's test, defibrinated sheep blood (20).



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