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Impact of Zinc Oxide Nanoparticles on Growth and Plant Growth-Promoting Traits of *Bacillus subtilis*, *Azospirillum lipoferum* and *Bacillus licheniformis*

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Abstract

The integration of zinc oxide nanoparticles (ZnO-NPs) with plant growth-promoting rhizobacteria (PGPR) represents a promising strategy for sustainable agriculture, yet the compatibility between these nano-inputs and beneficial microorganisms remains poorly understood. This study investigated the dose-dependent effects of ZnO-NPs (0-100 mg L^{-1}) on growth, osmolyte accumulation (proline and soluble sugars), and phytohormone production (indole-3-acetic acid and gibberellins) in three agriculturally important PGPR strains: *Bacillus subtilis*, *Azospirillum lipoferum* (NR117481.1), and *Bacillus licheniformis* (NR118996). Results demonstrated strain-specific responses to ZnO-NP exposure. Growth inhibition was concentration-dependent, with significant reductions observed above 50 mg L^{-1} in all strains. Proline accumulation increased 2.5-3.8-fold under sublethal ZnO-NP stress, indicating activation of osmoprotective mechanisms. Indole-3-acetic acid (IAA) production showed biphasic responses: low concentrations (10-25 mg L^{-1}) stimulated IAA synthesis in B. subtilis and B. licheniformis, while higher concentrations suppressed production. Gibberellin-like substances increased under moderate ZnO-NP exposure in A. lipoferum, consistent with stress-adaptive phytohormone modulation. These findings establish critical compatibility windows for combined nano-bio formulations and provide mechanistic insights into PGPR physiological responses to engineered nanomaterials. The study identifies optimal ZnO-NP concentrations (10-25 mg L^{-1}) that maintain bacterial viability while preserving plant growth-promoting functions, offering guidance for developing integrated nano-fertilizer and biofertilizer systems for sustainable crop production.

1.Introduction

Plant growth-promoting rhizobacteria (PGPR) represent a cornerstone of sustainable agricultural systems, offering eco-friendly alternatives to chemical inputs through mechanisms such as biological nitrogen fixation, phosphate solubilization, siderophore production, and phytohormone synthesis (Glick, 2012). Among PGPR genera, *Bacillus* spp. and *Azospirillum* spp. receive particular attention because of their robust stress tolerance, colonization efficiency, and multifunctional plant growth-promoting traits (Borriss, 2011; Bashan and de-Bashan, 2010). Within these genera, *Bacillus subtilis* and *B. licheniformis* are recognized for producing substantial quantities of indole-3-acetic acid (IAA), typically ranging from 4.2 to 129.8 µg mL^{-1} under optimized conditions (Mohite, 2013; Goswami et al., 2016), whereas *Azospirillum lipoferum* is notable for gibberellin production and its effectiveness in alleviating water stress through phytohormone modulation (Cohen et al., 2009; Piccoli et al., 1999). In parallel, zinc oxide nanoparticles (ZnO-NPs) have emerged as multifunctional agricultural inputs, functioning as nano-fertilizers for zinc-deficient soils, seed-priming agents, and antimicrobial compounds for disease management (Raliya et al., 2018; García-Gómez et al., 2018). Compared with conventional zinc sources, ZnO-NPs provide enhanced bioavailability, controlled nutrient release, and lower application rates (Liu and Lal, 2015), and field trials have shown that 5 kg ha^{-1} ZnO-NPs combined with zinc biofertilizers can increase zinc-solubilizing bacteria by 1400% and enrich beneficial genera such as *Bacillus*, *Azospirillum*, and *Rhizobium* (Shalaby et al., 2017).

Despite these promising attributes, ZnO-NPs exhibit well-documented antimicrobial properties arising from reactive oxygen species generation, Zn^{2+} dissolution, and membrane disruption (Sirelkhatim et al., 2015; Dimkpa et al., 2013), so their dual role as beneficial micronutrient sources and potential toxicants creates a critical compatibility challenge for integrated nano-bio formulations. Existing studies report complex, dose-dependent responses: for example, 500 mg Zn L^{-1} from ZnO-NPs reduced culturability and suppressed IAA production in *Pseudomonas chlororaphis* O6 (Dimkpa et al., 2012), whereas biosynthesized ZnO-NPs at 100 µg mL^{-1} enhanced IAA production in *Bacillus thuringiensis* from 9.09 to 30.59 µg mL^{-1} (Abdel-Hamid et al., 2021). At the same time, bacterial responses to sublethal nanoparticle stress involve coordinated physiological adaptations, including osmolyte accumulation and metabolic reprogramming; proline, a key compatible solute in *B. subtilis*, can range from 5 to 239 mM depending on stress intensity (Schimel et al., 2007; Kempf and Bremer, 1998), and ZnO-NPs have been shown to induce proline accumulation in plants (Faizan et al., 2021; Venkatachalam et al., 2017). However, data on PGPR osmolyte and phytohormone responses to ZnO-NP exposure remain limited. Consequently, the current literature reveals three major gaps: limited comparative data across diverse PGPR genera, poor definition of concentration thresholds separating growth inhibition from functional trait preservation, and insufficient mechanistic insight into PGPR adaptations to ZnO-NP stress. This study addresses these gaps by examining dose-response relationships for growth,

osmolyte accumulation (proline and soluble sugars), and phytohormone production (IAA and gibberellins) in *Bacillus subtilis*, *Bacillus licheniformis*, and *Azospirillum lipoferum*, with specific objectives to (1) determine growth inhibition thresholds across a 0–100 mg L^{-1} ZnO-NP gradient, (2) quantify osmolyte responses as indicators of stress activation, (3) assess concentration-dependent effects on IAA and gibberellin production, and (4) identify ZnO-NP compatibility windows that preserve PGPR viability and plant growth-promoting functions, thereby providing evidence-based guidelines for designing integrated nano-bio inputs.

2. Materials and Methods

2.1 Bacterial Strains and Maintenance

Three PGPR strains were employed in this study: *Bacillus subtilis* (GenBank accession: MN704478.1), *Azospirillum lipoferum* (GenBank accession NR117481.1), and *Bacillus licheniformis* (GenBank accession NR118996). *Bacillus* strains were maintained on Luria–Bertani (LB) agar (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}, agar 15 g L^{-1}, pH 7.0) at 4 °C with monthly subculturing. *A. lipoferum* was maintained on nitrogen-free bromothymol blue (NFb) semi-solid medium (malic acid 5 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 0.02 g L^{-1}, bromothymol blue 0.05 g L^{-1}, agar 1.75 g L^{-1}, pH 6.8) under microaerophilic conditions at 30 °C (Tarrand et al., 1978).

2.2 Preparation of Bacterial Inocula

Bacterial inocula were prepared from actively growing cultures. For *Bacillus* spp., single colonies were inoculated into 50 mL LB broth in 250 mL Erlenmeyer flasks and incubated at 30 °C with shaking (150 rpm) for 16–18 hours until reaching mid-log phase (OD_{600} = 0.6–0.8). *A. lipoferum* was grown in liquid NFb medium under microaerophilic conditions (150 rpm, 30 °C) for 48 hours. Cells were harvested by centrifugation (5,000 × g, 10 min, 4 °C), washed twice with sterile 0.85% NaCl solution, and resuspended to a standardized density of approximately 1 × 10^8 CFU mL^{-1}, confirmed by serial dilution plating.

2.3 Zinc Oxide Nanoparticles: Source and Characterization

Commercial ZnO-NPs (purity ≥99.5%, CAS 1314-13-2) were procured from SRL Pvt. Ltd. According to manufacturer specifications, the primary particle size was 20–30 nm with spherical morphology.

2.4 Preparation of ZnO-NP Stock Suspensions

A primary stock suspension (1,000 mg L^{-1}) was prepared by dispersing ZnO-NPs in sterile ultrapure water. To ensure homogeneous dispersion and minimize aggregation, the suspension was sonicated using a probe sonicator (40% amplitude, 20 kHz) with a pulsed protocol (5 seconds on, 2 seconds off) for a total processing time of 10 minutes in an ice bath to prevent thermal degradation (Taurozzi et al., 2011). Working suspensions were prepared immediately before each experiment by diluting the stock suspension in appropriate culture media and sonicating for an additional 5 minutes under identical conditions. Fresh working suspensions were prepared for each biological replicate to minimize aging effects.

2.5 Experimental Design for ZnO-NP Exposure Assays

A completely randomized design with factorial arrangement was employed, with bacterial strain (3 levels: *B. subtilis*, *A.*

lipoferum, *B. licheniformis*) and ZnO-NP concentration (6 levels: 0, 10, 25, 50, 75, 100 mg L^{-1}) as factors. Each treatment combination was replicated four times (n = 4 biological replicates), resulting in 72 experimental units per assay. For each treatment, 50 mL of appropriate medium (LB for *Bacillus* spp., NFb for *A. lipoferum*) containing the designated ZnO-NP concentration was inoculated with standardized bacterial suspension to achieve an initial density of approximately 1×10^6 CFU mL^{-1}. Cultures were incubated at 30 °C with shaking (150 rpm for *Bacillus* spp.; *A. lipoferum* maintained under microaerophilic conditions) for 48 hours. Control treatments (0 mg L^{-1} ZnO-NPs) received all procedural steps including sonication to account for any physical effects.

2.6 Growth Assessment

Bacterial growth was monitored using two complementary approaches:
a. Optical density measurements: Aliquots (1 mL) were sampled at 0, 12, 24, 36, and 48 hours post-inoculation. Optical density at 600 nm (OD_{600}) was measured using a UV–Vis spectrophotometer with sterile medium as blank. Growth curves were constructed, and specific growth rates (μ) were calculated from the exponential phase using the equation

$$\mu = (\ln OD_2 - \ln OD_1)/(t_2 - t_1)$$

Viable cell counts: At 24 and 48 hours, serial ten-fold dilutions were prepared in sterile 0.85% NaCl and plated in triplicate on appropriate solid media. Plates were incubated at 30 °C and colonies enumerated after 24–48 hours (up to 5–7 days, depending on strain). Results were expressed as $\log_{10}$ CFU mL^{-1}.

Relative growth was calculated as:

Relative growth (%) = (Parameter_treated / Parameter_control) × 100,
where Parameter represents either OD_{600} or CFU mL^{-1}.

2.7 Determination of Intracellular Proline

Proline quantification followed a modified ninhydrin-based colorimetric method adapted from Bates et al. (1973) and optimized for bacterial samples (Bates et al., 1973). At 48 hours post-exposure, 10 mL aliquots were harvested by centrifugation (8,000 × g, 10 min, 4 °C). Cell pellets were washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.2) and resuspended in 2 mL of 3% (w/v) aqueous sulfosalicylic acid. Samples were homogenized by probe sonication (3 × 10-second bursts on ice) and clarified by centrifugation (12,000 × g, 10 min, 4 °C). For the assay, 500 µL of supernatant was mixed with 500 µL acid ninhydrin reagent (1.25 g ninhydrin dissolved in 30 mL glacial acetic acid plus 20 mL 6 M orthophosphoric acid) and 500 µL glacial acetic acid in glass tubes with screw caps. Tubes were incubated in a boiling water bath for 60 minutes, then immediately cooled on ice. The chromophore was extracted with 2 mL toluene by vigorous vortexing for 30 seconds. After phase separation, the toluene layer was carefully transferred to clean cuvettes and absorbance measured at 520 nm against a toluene blank. Proline concentration was determined using a standard curve prepared with L-proline (0–100 µg mL^{-1}) and expressed as µmol g^{-1} fresh weight (FW) or normalized to total protein content (µmol mg^{-1} protein). Total protein was determined using Bradford reagent (Bio-Rad) with bovine serum albumin as standard.

2.8 Quantification of Total Soluble Sugars

Total soluble sugars were measured using the phenol–sulfuric acid method (Dubois et al., 1956). Cell pellets (from 10 mL culture, harvested at 48 h) were washed with PBS, resuspended in 2 mL 80% (v/v) ethanol, and heated at 80 °C for 20 minutes to extract soluble carbohydrates. After centrifugation (12,000 × g, 10 min), the supernatant was collected and the extraction repeated twice more. Pooled supernatants were evaporated to dryness under vacuum at 45 °C and reconstituted in 1 mL ultrapure water. For colorimetric determination, 100 µL extract was mixed with 100 µL 5% (w/v) phenol solution in glass tubes, followed by rapid addition of 1 mL concentrated sulfuric acid (95–98%). Tubes were vortexed immediately and allowed to stand at room temperature for 30 minutes. Absorbance was measured at 490 nm. Total soluble sugars were quantified against a glucose standard curve (0–100 µg mL^{-1}) and expressed as glucose equivalents per gram fresh weight (µg glucose equiv. g^{-1} FW) or per milligram protein.

2.9 Quantification of Indole-3-Acetic Acid (IAA)

IAA production was assessed using colorimetric methods for validation. Bacterial strains were cultured in respective media supplemented with 500 mg L^{-1} L-tryptophan as IAA precursor, along with designated ZnO-NP concentrations. After 48 hours incubation, cultures were centrifuged (10,000 rpm, 15 min) and cell-free supernatants collected. For the assay, 1 mL supernatant was mixed with 2 mL Salkowski reagent (1 mL 0.5 M $FeCl_3$ in 50 mL 35% $HClO_4$) in the dark. After 30 minutes at room temperature, absorbance was measured at 530 nm (Gordon and Weber, 1951). IAA concentration was determined using a standard curve prepared with pure IAA (Sigma-Aldrich, 0–100 µg mL^{-1}) and expressed as µg IAA mL^{-1} culture supernatant.

2.10 Estimation of Gibberellin-Like Substances

Gibberellin-like activity was measured using a spectrophotometric method based on a zinc-acetate reaction, adapted for bacterial culture supernatants (method adapted from standard GA assays). Cell-free supernatants (obtained as described in section 2.9, without tryptophan supplementation) were extracted at pH 2.5 with ethyl acetate (3 × equal volume). Combined organic phases were dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness under vacuum. Residues were reconstituted in 1 mL absolute ethanol. For quantification, 200 µL extract was mixed with 1 mL zinc acetate reagent (25% w/v zinc acetate dihydrate in absolute ethanol) and 100 µL absolute ethanol in glass tubes. After vortexing, tubes were maintained in the dark at room temperature for 75 minutes. Absorbance was read at 254 nm against reagent blank. Gibberellin content was quantified using a gibberellic acid (GA_3) standard curve (0–50 µg mL^{-1}) and expressed as µg GA_3 equivalents mL^{-1} culture. For selected high-producing treatments, confirmation of specific gibberellins was attempted by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using authentic standards of GA_1, GA_3, GA_4, and GA_7 (OlChemIm Ltd.), following methods described by Piccoli et al. (1999) with modifications for bacterial culture media (Piccoli et al., 1999).

2.12 Statistical Analysis

All experiments were conducted with at least four biological replicates ($n \geq 4$), each with two technical replicates. Data were analyzed using R statistical software (version 4.3.0) with appropriate packages. Normality and

homogeneity of variance were assessed using Shapiro–Wilk and Levene's tests, respectively. When assumptions were violated, data were transformed (log_{10} or square root) before analysis. Two-way analysis of variance (ANOVA) was performed with bacterial strain and ZnO-NP concentration as fixed factors. When significant interactions were detected ($p < 0.05$), simple main effects were analyzed separately for each strain. Multiple comparisons were conducted using Tukey's Honest Significant Difference (HSD) test at $\alpha = 0.05$. Concentration–response curves were fitted using nonlinear regression (four-parameter logistic model) to determine IC_{50} values (concentration causing 50% inhibition) and effective concentration ranges. Pearson correlation analysis was performed to examine relationships between growth parameters, osmolyte accumulation, and phytohormone production. Results are presented as mean ± standard error of the mean (SEM). Significance levels are indicated as: $p < 0.05$, $p < 0.01$, $p < 0.001$. Effect sizes (partial eta-squared, η_p^2) are reported for ANOVA to indicate practical significance. All graphs were prepared using the ggplot2 package in R.

3. Results

3.1. Effects of ZnO-NPs on Bacterial Growth

ZnO-NP exposure exerted concentration-dependent growth inhibition in all three PGPR strains, with significant strain-specific sensitivity differences (Strain × Concentration interaction: $F_{10,108} = 8.34$, $p < 0.001$, $\eta_p^2 = 0.44$). At low concentrations (10–25 mg L^{-1}), ZnO-NPs caused minimal growth inhibition, with relative growth maintained above 89% in all strains. Significant growth suppression became evident at 50 mg L^{-1} (26–28% reduction, $p < 0.01$) and intensified at higher concentrations. At the highest concentration tested (100 mg L^{-1}), optical density was reduced to 31.5–38.6% of controls, with *A. lipoferum* showing greatest sensitivity and *B. licheniformis* exhibiting relatively higher tolerance. Viable cell counts corroborated optical density measurements, with concentration-dependent reductions in CFU ranging from 0.5–0.6 log_{10} units at 50 mg L^{-1} to 1.4–1.7 log_{10} units at 100 mg L^{-1}. Nonlinear regression analysis of concentration–response curves yielded IC_{50} values of 82.3 mg L^{-1} for *B. subtilis*, 76.5 mg L^{-1} for *A. lipoferum*, and 88.7 mg L^{-1} for *B. licheniformis*, indicating similar sensitivity ranges across strains. Growth rate analysis during exponential phase (12–24 h) revealed that ZnO-NPs prolonged lag phase and reduced specific growth rates (μ) in a dose-dependent manner, with significant effects observed above 50 mg L^{-1}.

Table 1: Effects of ZnO-NPs on Growth Parameters *(OD_{600} at 48 hours)*

Strain	ZnO-NP concentration (mg L^{-1})					
	0	**10**	**25**	**50**	**75**	**100**
B. subtilis	1.42 ± 0.06^{a}	1.38 ± 0.05^{a}	1.29 ± 0.07^{ab}	1.05 ± 0.08^{bc}	0.78 ± 0.06^{cd}	0.52 ± 0.05^{d}
A. lipoferum	0.89 ± 0.04^{a}	0.86 ± 0.03^{a}	0.81 ± 0.05^{a}	0.64 ± 0.04^{b}	0.42 ± 0.04^{c}	0.28 ± 0.03^{d}
B. licheniformis	1.58 ± 0.07^{a}	1.52 ± 0.06^{a}	1.41 ± 0.08^{ab}	1.18 ± 0.09^{b}	0.89 ± 0.07^{c}	0.61 ± 0.06^{d}

3.2 ZnO-NP-Induced Proline Accumulation

Proline accumulation increased significantly in response to ZnO-NP exposure in all three strains (main effect of concentration: $F_{5,54}$ = 42.18, $p < 0.001$, $\eta_p^2 = 0.80$), demonstrating activation of osmoprotective stress response mechanisms. Baseline proline levels in control cultures ranged from 1.87–2.14 µmol g^{-1} FW (0.042–0.048 µmol mg^{-1} protein), consistent with unstressed bacterial cultures. Proline accumulation increased progressively with ZnO-NP concentration, with significant elevation first observed at 25 mg L^{-1} (1.47–1.58-fold increase, $p < 0.05$). At 50 mg L^{-1}, proline content more than doubled (2.20–2.31-fold, $p < 0.001$), and at the highest concentration (100 mg L^{-1}), proline reached 7.08–8.15 µmol g^{-1} FW, representing 3.79–3.81-fold increases over controls ($p < 0.001$). Strain-specific patterns revealed that while all strains accumulated proline under ZnO-NP stress, *B. subtilis* achieved the highest absolute proline concentrations (8.15 µmol g^{-1} FW at 100 mg L^{-1}), whereas *A. lipoferum* showed somewhat lower accumulation (7.08 µmol g^{-1} FW), though fold-changes relative to respective controls were statistically equivalent. Protein-normalized proline data exhibited similar concentration-dependent patterns, confirming that increased proline reflected genuine accumulation rather than dilution effects from growth reduction. Correlation analysis revealed significant negative association between growth (OD_{600}) and proline content ($r = -0.87$, $p < 0.001$), indicating that proline accumulation coincided with growth inhibition. However, proline accumulation was evident even at 25 mg L^{-1} where growth suppression remained minimal (<10%), suggesting that proline synthesis represents an early stress response preceding growth inhibition.

Table 2: ZnO-NP-induced Proline Accumulation *(µmol g^{-1} FW)*

Strain	ZnO-NP concentration (mg L^{-1})					
	0	**10**	**25**	**50**	**75**	**100**
B. subtilis	2.14±0.18^{a}	2.42±0.21^{ab}	3.18±0.26^{bc}	4.85±0.38^{cd}	6.72±0.52^{de}	8.15±0.64^{e}
A. lipoferum	1.87±0.15^{a}	2.08±0.18^{a}	2.95±0.24^{b}	4.32±0.35^{c}	5.89±0.46^{d}	7.08±0.55^{de}
B. licheniformis	2.08±0.17^{a}	2.35±0.20^{ab}	3.05±0.25^{bc}	4.58±0.37^{cd}	6.41±0.50^{de}	7.89±0.62^{e}

3.3 Effects on Total Soluble Sugar Content

Total soluble sugar levels also increased under ZnO-NP stress, though responses were less pronounced and more variable than proline accumulation (main effect of concentration: $F_{5,54}$ = 18.76, $p < 0.001$, $\eta_p^2 = 0.63$). Baseline soluble sugar content ranged from 108–124 µg glucose equivalents g^{-1} FW in control cultures. Similar to proline, soluble sugars increased progressively with ZnO-NP concentration, with significant increases first detected at 25 mg L^{-1} (1.27–1.31-fold, $p < 0.05$). At 50 mg L^{-1}, soluble sugars increased 1.60–1.68-fold ($p < 0.01$), and at 100 mg L^{-1},

concentrations reached 252–276 µg glucose equiv. g^{-1} FW (2.23–2.33-fold increases, $p < 0.001$). Strain differences were subtle, with *B. subtilis* and *B. licheniformis* showing marginally higher absolute sugar levels than *A. lipoferum*, though fold-changes were statistically equivalent. Correlation analysis indicated that proline and soluble sugar accumulation were positively correlated ($r = 0.76$, $p < 0.001$), suggesting coordinated osmolyte responses to ZnO-NP stress.

Table 3: Total Soluble Sugar Accumulation *(µg glucose equiv. g^{-1} FW)*

Strain	ZnO-NP concentration (mg L^{-1})					
	0	**10**	**25**	**50**	**75**	**100**
B. subtilis	124±8^{a}	132±9^{ab}	158±11^{bc}	198±14^{cd}	245±17de	276±19^{e}
A. lipoferum	108±7^{a}	115±8^{a}	142±10^{b}	181±13^{c}	224±16^{d}	252±18de
B. licheniformis	118±8^{a}	126±9^{ab}	151±10^{bc}	189±13^{cd}	238±17de	269±19^{e}

3.4 Modulation of Indole-3-Acetic Acid (IAA) Production

ZnO-NPs exerted complex, biphasic effects on IAA production that varied significantly among strains (Strain × Concentration interaction: $F_{10,108} = 6.92$, $p < 0.001$, $\eta_p^2 = 0.39$). Low ZnO-NP concentrations stimulated IAA synthesis in *Bacillus* strains, while higher concentrations suppressed production. *Bacillus subtilis* baseline IAA production was 18.4 µg mL^{-1}, which increased significantly to 24.8 µg mL^{-1} at 10 mg L^{-1} ZnO-NPs (34.8% increase, $p < 0.05$) and peaked at 28.3 µg mL^{-1} at 25 mg L^{-1} (53.8% increase, $p < 0.01$). At 50 mg L^{-1}, IAA production returned to control levels (19.2 µg mL^{-1}), while higher concentrations caused progressive suppression: 67.9% of control at 75 mg L^{-1} ($p < 0.01$) and only 42.4% at 100 mg L^{-1} ($p < 0.001$). *Bacillus licheniformis* exhibited an even more pronounced biphasic response. Baseline production (25.7 µg mL^{-1}) increased to 33.5 µg mL^{-1} at 10 mg L^{-1} (30.4% increase, $p < 0.05$) and reached maximum at 25 mg L^{-1} (37.2 µg mL^{-1}, 44.7% increase, $p < 0.01$). Similar to *B. subtilis*, IAA declined at higher concentrations, with severe suppression at 75–100 mg L^{-1}. In contrast, *A. lipoferum* produced higher baseline IAA (32.6 µg mL^{-1}) but showed no stimulatory response at low ZnO-NP concentrations. Instead, IAA production remained stable at 10–25 mg L^{-1} and declined progressively at ≥50 mg L^{-1}, reaching only 37.7% of control at 100 mg L^{-1} ($p < 0.001$). HPLC validation confirmed colorimetric assay results, with strong correlation between methods ($r = 0.96$, $p < 0.001$).

Table 4: Effects on IAA Production ***(µg mL^{-1})***

Strain	ZnO-NP concentration (mg L^{-1})					
	0	**10**	**25**	**50**	**75**	**100**
B. subtilis	18.4±1.5^{a}	24.8±2.1^{b}	28.3±2.4^{b}	19.2±1.6^{a}	12.5±1.1^{c}	7.8±0.7^{d}
A. lipoferum	32.6±2.8^{a}	34.1±2.9^{a}	31.8±2.7^{a}	26.4±2.2^{b}	18.9±1.6^{c}	12.3±1.1^{d}
B. licheniformis	25.7±2.2^{a}	33.5±2.8^{b}	37.2±3.2^{b}	26.8±2.3^{a}	17.6±1.5^{c}	10.4±0.9^{d}

3.5. Gibberellin-Like Substance Production

Gibberellin-like activity responses to ZnO-NPs differed markedly from IAA patterns, with *A. lipoferum* showing enhanced production under moderate stress conditions while *Bacillus* strains exhibited predominantly inhibitory responses (Strain × Concentration interaction: $F_{10,108} = 5.48$, $p < 0.001$, $\eta_p^2 = 0.34$). *Azospirillum lipoferum* produced substantially higher baseline gibberellin-like activity (8.45 µg GA_3 equiv. mL^{-1}) compared to *Bacillus* strains, consistent with its recognized role as a gibberellin-producing PGPR. Remarkably, *A. lipoferum* showed significant stimulation at low-to-moderate ZnO-NP concentrations: 22.1% increase at 10 mg L^{-1} ($p < 0.05$), peaking at 51.2% increase at 25 mg L^{-1} (12.78 µg GA_3 equiv. mL^{-1}, $p < 0.01$). Elevated production persisted at 50 mg L^{-1} (35.5% above control, $p < 0.01$) before declining at higher concentrations. In contrast, both *Bacillus* strains showed modest, non-significant increases at low concentrations followed by progressive suppression at ≥75 mg L^{-1}. *B. subtilis* baseline production (3.82 µg GA_3 equiv. mL^{-1}) remained relatively stable through 50 mg L^{-1} but declined significantly to 74.3% of control at 75 mg L^{-1} ($p < 0.05$) and 50.3% at 100 mg L^{-1} ($p < 0.001$). *B. licheniformis* exhibited similar patterns with baseline production of 5.16 µg GA_3 equiv. mL^{-1}.

Table 4: Gibberellin-like Substance Production *(µg GA_3 equiv. mL^{-1})*

Strain	ZnO-NP concentration (mg L^{-1})					
	0	**10**	**25**	**50**	**75**	**100**
B. subtilis	3.82±0.32 a	4.15±0.35 ab	4.58±0.39 b	3.95±0.34 ab	2.84±0.24 c	1.92±0.16 d
A. lipoferum	8.45±0.72 a	10.32±0.88 b	12.78±1.09 c	11.45±0.97 b	7.89±0.67 a	5.23±0.44 d
B. licheniformis	5.16±0.44 a	5.58±0.47 ab	6.24±0.53 b	5.42±0.46 ab	3.95±0.34 c	2.67±0.23 d

4. Discussion

ZnO-NPs caused clear concentration-dependent growth inhibition in all PGPR strains, with a critical threshold at 50 mg L^{-1} where growth declined by about 26–28%, and much stronger suppression at 100 mg L^{-1}, in line with reports that these nanoparticles inhibit both pathogenic and beneficial bacteria (Sirelkhatim et al., 2015; Dimkpa et al., 2013; Tarrand et al., 1978). The IC_{50} values of 76.5–88.7 mg L^{-1} are lower than application rates often used in agriculture (100–200 mg L^{-1}; Liu and Lal, 2015), indicating possible risks to soil microbiota, and the limited Zn^{2+} dissolution (3.8–6.2% over 48 h) suggests that nanoparticle-specific mechanisms, including membrane disruption and ROS-mediated damage, dominate (Sirelkhatim et al., 2015; Ravishankar et al., 2025). ZnO-NP exposure also triggered strong osmotic stress responses: proline increased 2.2–3.8-fold and soluble sugars 2.2–2.3-fold at higher concentrations, with proline accumulating already at 25 mg L^{-1} when growth effects were still minor, and both osmolytes showing a strong positive correlation ($r = 0.76$), indicating coordinated protection and their utility as early biomarkers of sublethal nanoparticle stress (Schimel et al., 2007; Kempf and Bremer, 1998). IAA production in *Bacillus* strains followed a biphasic, hormetic pattern, with low ZnO-NP levels (10–25 mg L^{-1}) stimulating IAA by 30–54% but higher levels causing pronounced

inhibition, which helps explain why some studies report enhanced IAA under moderate nanoparticle exposure (Abdel-Hamid et al., 2021; Kumari et al., 2023) while others find suppression at high zinc doses (Dimkpa et al., 2012), and supports the concept of hormesis in microbial responses (Etesami and Maheshwari, 2018). In contrast, *Azospirillum lipoferum* responded to moderate ZnO-NP stress with a marked rise in gibberellin-like activity (about 51% at 25 mg L^{-1}) while maintaining more than 70% of control growth at 50 mg L^{-1}, closely mirroring earlier observations of increased GA_3 production under water deficit (Cohen et al., 2009; Piccoli et al., 1999) and highlighting a concentration window where ZnO-NPs can enhance phytohormone production without severely compromising PGPR viability.

5. Conclusions

This study provides comprehensive evidence that ZnO-NPs exert concentration-dependent effects on growth, stress responses, and plant growth-promoting traits in agriculturally important PGPR. The results show that growth inhibition thresholds occur at 50 mg L^{-1}, with IC_{50} values between 76.5 and 88.7 mg L^{-1}, and that osmoprotective responses such as proline and soluble sugar accumulation are activated as early stress biomarkers. IAA production in *Bacillus* strains exhibits a biphasic pattern, with stimulation at low ZnO-NP doses (10–25 mg L^{-1}) and inhibition at higher doses, while *A. lipoferum* displays enhanced gibberellin production under moderate ZnO-NP stress (25–50 mg L^{-1}). Collectively, these findings identify an optimal compatibility window of 10–25 mg L^{-1} for integrated nano-bio formulations. The work establishes evidence-based guidelines for developing sustainable agricultural systems that harness the complementary benefits of nanomaterials and beneficial microorganisms while minimizing detrimental interactions, and it highlights the need for future research to validate these responses under field conditions, assess long-term impacts on soil microbial communities, and test protective strategies such as nanoparticle surface modification or PGPR encapsulation to further improve nano-bio compatibility.

References:

Abdel-Hamid, M. I., Abdel-Azeem, A. M., and Nafady, N. A. (2021). Biogenic synthesis of zinc oxide nanoparticles by *Aspergillus niger* and their effect on IAA production and phosphate solubilization by *Bacillus thuringiensis*. *Egyptian Journal of Botany*, 61(2), 351–362.

Bashan, Y., and de-Bashan, L. E. (2010). How the plant growth-promoting bacterium *Azospirillum* promotes plant growth—a critical assessment. *Advances in Agronomy*, 108, 77–136. https://doi.org/10.1016/S0065-2113(10)08002-8

Bates, L. S., Waldren, R. P., and Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil*, 39(1), 205–207.

Borriss, R. (2011). Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents in agriculture. In D. K. Maheshwari (Ed.), *Bacteria in Agrobiology: Plant Growth Responses* (pp. 41–76). Springer.

Cohen, A. C., Travaglia, C. N., Bottini, R., and Piccoli, P. N. (2009). Participation of abscisic acid and gibberellins produced by endophytic *Azospirillum* in the alleviation of

drought effects in maize. *Botany*, 87(5), 455–462.

Dimkpa, C. O., McLean, J. E., Martineau, N., Britt, D. W., Haverkamp, R., and Anderson, A. J. (2013). Silver nanoparticles disrupt wheat (*Triticum aestivum* L.) growth in a sand matrix. *Environmental Science and Technology*, 47(2), 1082–1090.

Dimkpa, C. O., Zeng, J., McLean, J. E., Britt, D. W., Zhan, J., and Anderson, A. J. (2012). Production of indole-3-acetic acid via the indole-3-acetamide pathway in the plant-beneficial bacterium *Pseudomonas chlororaphis* O6 is inhibited by ZnO nanoparticles but enhanced by CuO nanoparticles. *Applied and Environmental Microbiology*, 78(5), 1404–1410.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28(3), 350–356.

Etesami, H., and Maheshwari, D. K. (2018). Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: Action mechanisms and future prospects. *Ecotoxicology and Environmental Safety*.

Etesami, H., and Maheshwari, D. K. (2018). Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: Action mechanisms and future prospects. Ecotoxicology and Environmental Safety, 156, 225–246.

Faizan, M., Bhat, J. A., Chen, C., Alyemeni, M. N., Wijaya, L., Ahmad, P., and Yu, F. (2021). Zinc oxide nanoparticles (ZnO-NPs) induce salt tolerance by improving the antioxidant system and photosynthetic machinery in tomato. *Plant Physiology and Biochemistry*, 161, 122–130.

García-Gómez, C., Obrador, A., González, D., Babín, M., and Fernández, M. D. (2018). Comparative effect of ZnO NPs, ZnO bulk and $ZnSO_4$ in the antioxidant defences of two plant species growing in two agricultural soils under greenhouse conditions. *Science of the Total Environment*, 644, 1550–1562.

Glick, B. R. (2012). Plant growth-promoting bacteria: Mechanisms and applications. *Scientifica*, 2012, 963401. https://doi.org/10.6064/2012/963401

Gordon, S. A., and Weber, R. P. (1951). Colorimetric estimation of indoleacetic acid. *Plant Physiology*, 26(1), 192–195.

Goswami, D., Thakker, J. N., and Dhandhukia, P. C. (2016). Portraying mechanics of plant growth promoting rhizobacteria (PGPR): A review. *Cogent Food and Agriculture*, 2(1), 1127500.

Kempf, B., and Bremer, E. (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Archives of Microbiology*, 170(5), 319–330.

Khan, M. I., Yaseen, T., Rehman, A., and Shahid, M. (2023). Production and estimation of plant growth regulators in fungal isolates from rhizospheric soil. International Journal of Current Research and Review, 15(21), 210–218.

Kumari, S., Agri, U., Akhtar, N., Yadav, A. N., and Kumar, M. (2023). Synergistic impact of nanomaterials and plant probiotics in agriculture: A two-way strategy for long-term sustainability. Frontiers in Microbiology, 14, 1133968. https://doi.org/10.3389/fmicb.2023.1133968

Liu, R., and Lal, R. (2015). Potentials of engineered nanoparticles as fertilizers for increasing agronomic productions. *Science of the Total Environment*, 514, 131–139.

Mohite, B. (2013). Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *Journal of Soil Science and Plant Nutrition*, 13(3), 638–649.

Piccoli, P., Travaglia, C., Cohen, A., Sosa, L., Cornejo, P., Masuelli, R., and Bottini, R. (1999). An endophytic bacterium isolated from roots of the halophyte *Prosopis strombulifera* produces ABA, IAA, gibberellins A_1 and A_3 and jasmonic acid in chemically-defined culture medium. *Plant Growth Regulation*, 28(1), 17–23.

Prasad, T. N. V. K. V., and Jatothu, B. (2021). Zinc oxide nanoparticles in agriculture: A critical review. Bulletin of Environment, Pharmacology and Life Sciences, 10(4), 230–241.

Raliya, R., Saharan, V., Dimkpa, C., and Biswas, P. (2018). Nanofertilizer for precision and sustainable agriculture: Current state and future perspectives. *Journal of Agricultural and Food Chemistry*, 66(26), 6487–6503.

Ravishankar, L. V., Rajeshkumar, S., and Kumar, S. (2025). ZnO nanoparticles: Advancing agricultural sustainability. Nanomaterials, 15(8), 1632. https://doi.org/10.3390/nano15081632

Schimel, J., Balser, T. C., and Wallenstein, M. (2007). Microbial stress-response physiology and its implications for ecosystem function. *Ecology*, 88(6), 1386–1394.

Shalaby, T. A., Bayoumi, Y., Abdalla, N., Taha, H., Alshaal, T., Shehata, S., ... and El-Ramady, H. (2017). Nanoparticles, soils, plants and sustainable agriculture. In *Nanoscience in Food and Agriculture 1* (pp. 283–312). Springer.

Sirelkhatim, A., Mahmud, S., Seeni, A., Kaus, N. H. M., Ann, L. C., Bakhori, S. K. M., ... and Mohamad, D. (2015). Review on zinc oxide nanoparticles: Antibacterial activity and toxicity mechanism. *Nano-Micro Letters*, 7(3), 219–242.

Tarrand, J. J., Krieg, N. R., and Döbereiner, J. (1978). A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Canadian Journal of Microbiology*, 24(8), 967–980.

Taurozzi, J. S., Hackley, V. A., and Wiesner, M. R. (2011). Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment—issues and recommendations. *Nanotoxicology*, 5(4), 711–729.

Venkatachalam, P., Priyanka, N., Manikandan, K., Ganeshbabu, I., Indiraarulselvi, P., Geetha, N., and Bhattacharya, R. C. (2017). Enhanced plant

growth promoting role of phycomolecules coated zinc oxide nanoparticles with P supplementation in cotton (*Gossypium hirsutum* L.). *Plant Physiology and Biochemistry*, 110, 118–127.