



Article

Commercial Zinc Oxide Nanoparticles: Mechanistic Investigation into the Bacterial Leaf Blight Pathogen of Rice and Evaluation of Their Biocompatibility

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Abstract

Bacterial leaf blight (BLB), a destructive disease of rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), continues to limit rice productivity worldwide. Although biologically synthesized zinc oxide nanoparticles (ZnO NPs) have been extensively investigated, knowledge regarding the antibacterial activity and biocompatibility of commercially available ZnO NPs is still limited. In this study, commercial ZnO NPs were systematically characterized and evaluated for their antibacterial mechanisms and biocompatibility in mammalian cells. FE-SEM and TEM analyses revealed irregular polyhedral, hexagonal, and short rod-like morphologies with an average particle size of ~33 nm, consistent with crystallite sizes estimated by XRD. The nanoparticles exhibited pronounced antibacterial activity against Xoo, with a minimum inhibitory concentration (MIC) of 16 µg/mL and a clear dose-dependent response. Mechanistic assays confirmed multifaceted bactericidal actions involving membrane disruption, ROS generation, Zn²⁺ release, and ultrastructural damage. Biocompatibility testing in human dermal fibroblasts showed enhanced proliferation at 8–32 µg/mL, no cytotoxicity up to 256 µg/mL, and reduced viability only at ≥512 µg/mL. These findings represent the first mechanistic evaluation of commercial ZnO NPs against Xoo, together with cytotoxicity assessment in mammalian cells, highlighting their structural distinctness and dual functionality that combine potent antibacterial activity with minimal mammalian cytotoxicity. Overall, the results underscore their potential as safe nanobiocontrol agents for sustainable rice disease management.

Keywords: zinc oxide nanoparticles; *Xanthomonas oryzae* pv. *oryzae*; antibacterial mechanism; reactive oxygen species; cytotoxicity; nanobiocontrol



Academic Editors: Heyou Han, João Rodrigues, Mohamed F. Foda and Lok. R. Pokhrel

Received: 6 October 2025

Revised: 27 October 2025

Accepted: 10 November 2025

Published: 13 November 2025

Citation: Jaiyan, T.; Rangsrirak, P.; Rahaeng, K.; Maensiri, D.; Mahakham, W. Commercial Zinc Oxide Nanoparticles: Mechanistic Investigation into the Bacterial Leaf Blight Pathogen of Rice and Evaluation of Their Biocompatibility. *Appl. Nano* **2025**, *6*, 26. <https://doi.org/10.3390/applnano6040026>

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1. Introduction

Rice (*Oryza sativa* L.) remains the principal dietary source for over three billion people and contributes roughly one-fifth of global caloric intake [1]. Most of the world's rice is produced in Asia, where it plays a central role in ensuring both food security and the livelihoods of rural populations [2]. However, the sustainability of rice production is increasingly threatened by bacterial leaf blight (BLB), a devastating vascular disease caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) [3,4]. This pathogen is recognized as one of the most destructive bacterial agents of cereal crops, with outbreaks capable of reducing yields

by 20–70%, depending on cultivar susceptibility and prevailing environmental factors [5]. The pathogen invades rice leaves through hydathodes and wounds, colonizing the xylem vessels and causing progressive wilting and necrosis [4,6]. Traditional management strategies relying on antibiotics and copper-based bactericides have become less effective due to increasing pathogen resistance and environmental concerns [6,7]. Biological alternatives such as beneficial microbes and bacteriophages have shown potential, yet their performance remains inconsistent under field conditions [8,9]. These limitations highlight the need for more reliable, sustainable, and reproducible disease-control strategies.

Nanotechnology has opened new avenues for crop disease management by improving the stability, targeted transport, and gradual release of bioactive agents [10]. Among engineered nanomaterials, zinc oxide nanoparticles (ZnO NPs) are particularly notable for their strong antibacterial activity, relatively low toxicity, and versatile use in both agricultural and biomedical applications [11,12]. The synthesis method, whether physical, chemical, or biological, plays a decisive role in determining key physicochemical properties such as particle size, morphology, and crystallinity, which ultimately influence their biological performance [13]. In particular, biologically mediated or green synthesis using plant extracts has gained popularity for its environmental friendliness, low cytotoxicity, and high biocompatibility [5]. Green-synthesized ZnO NPs are typically coated with phytochemical capping agents that improve stability and biological interactions by introducing hydroxyl and phenolic groups on the nanoparticle surface, which facilitate reactive oxygen species (ROS) generation, mainly hydroxyl and superoxide radicals responsible for damaging bacterial membranes and macromolecules, thereby providing high photocatalytic efficiency and broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria [14]. They have also shown strong inhibition of phytopathogenic microorganisms infecting economically important crops [5,15–20], while foliar applications have improved rice seedling vigor and grain quality under stress conditions [21]. Although green-synthesized ZnO NPs have demonstrated great potential, differences in plant-derived reducing agents and synthesis parameters can result in variations in particle properties. This diversity highlights the need for complementary approaches, such as the use of commercially standardized ZnO NPs, to achieve reproducible outcomes and enable broader agricultural applications.

In contrast, commercially produced ZnO NPs are synthesized through standardized physical and chemical methods that allow precise control over reaction conditions, scalability, and product uniformity [13,22]. These advantages make them attractive for practical agricultural use, where reproducibility, availability, and regulatory compliance are essential considerations. Commercial nanopowders are widely available and have been used in diverse applications ranging from food packaging and cosmetics to crop protection [23,24]. Surprisingly, despite these expected benefits, previous reports have revealed notable batch-to-batch variability even within the same catalog product (e.g., Sigma-Aldrich Cat. No. 544906). For example, Konor et al. [25] described quasi-spherical ZnO NPs averaging about 45 nm, whereas Orozco-Messana [26] reported aggregated equiaxial particles with a crystallite size of approximately 75 nm. Interestingly, our preliminary SEM and TEM observations of the same catalog product also indicated morphological differences compared with these earlier reports, emphasizing the need for detailed physicochemical and biological evaluation. Such unexpected variations may arise from subtle differences in precursor purity, synthesis temperature, or post-synthetic processing during large-scale manufacturing. These findings suggest that even standardized commercial nanopowders should undergo comprehensive physicochemical characterization before biological assessment.

To ensure the safe and responsible use of commercially manufactured ZnO nanoparticles (ZnO NPs) across agricultural and biomedical domains, it is essential to assess both

their antibacterial performance and potential effects on mammalian cells. While the antimicrobial activity of ZnO NPs has been extensively characterized, relatively few studies have explored their safety toward mammalian systems. Experimental investigations in both animal and cell-based models are indispensable for understanding how nanoparticles may influence biological processes and pose possible health risks [27]. Among these, *in vitro* assays provide a controlled and reproducible platform to examine nanoparticle–cell interactions, including alterations in cellular morphology, adhesion behavior, and proliferation patterns [27]. The skin is one of the most common exposure routes for metal oxide nanoparticles because ZnO NPs are widely incorporated into topical formulations such as sunscreens, wound dressings, and cosmetics [28]. Once deposited on the skin, nanoparticles can traverse the epidermal layer and reach the dermis, where human dermal fibroblasts (HDFs) represent the predominant structural and metabolically active cell type [29]. HDFs play key roles in collagen synthesis, extracellular matrix remodeling, and tissue repair [29], making them particularly relevant for biocompatibility evaluation. Moreover, *in vitro* studies have shown that both human periodontal ligament and dermal fibroblasts exhibit concentration- and time-dependent cytotoxic effects following ZnO NP exposure, highlighting fibroblasts as reliable *in vitro* models for evaluating nanoparticle-induced cellular toxicity [27]. In addition, ZnO NPs can induce oxidative stress and apoptosis in HDFs through p53–p38 MAPK activation [30], while zinc-based compounds elicit similar stress-response profiles [31]. At low, non-toxic concentrations, however, ZnO NPs can promote fibroblast migration and proliferation, thereby facilitating wound repair [32]. This bidirectional behavior underscores the need to define concentration thresholds that distinguish beneficial from adverse responses. Therefore, HDFs were selected in this study as a physiologically relevant *in vitro* model for early screening of ZnO NP biosafety prior to *in vivo* or field applications.

While this study is based on controlled *in vitro* assays, the findings provide a foundational reference for subsequent field-scale validations. The outcomes of this study aim to establish a reproducible reference baseline for interpreting biological responses and to provide practical insights into the safe and effective use of commercial ZnO NPs in both agricultural and biomedical contexts, particularly as sustainable nanobiocontrol agents for bacterial leaf blight management in rice and as biocompatible materials for potential dermal exposure applications.

2. Materials and Methods

2.1. Chemicals and Commercial ZnO NPs

Commercial zinc oxide nanopowder (ZnO NPs) (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. 544906, <100 nm) was used throughout this work as the test material. According to the supplier, this product is provided as a nanopowder with a reported particle size below 100 nm, a specific surface area of 10–25 m² g^{−1}, and zinc content of approximately 79%. All other reagents were of analytical grade. Sterile deionized water was used for nanoparticle dispersion and dilution in all assays.

2.2. Characterization of ZnO NPs

The commercial ZnO NPs were examined for morphology, particle size, and crystallinity prior to biological testing.

For field-emission scanning electron microscopy (FE-SEM), the nanopowder was dispersed in sterile deionized water using brief ultrasonic agitation. A drop of the suspension was transferred onto carbon tape mounted on aluminum stubs and allowed to air-dry. Imaging and elemental analysis were performed using a JSM-7800F field-emission

SEM (JEOL Ltd., Tokyo, Japan) equipped with an energy-dispersive X-ray spectroscopy (EDX) detector.

For transmission electron microscopy (TEM), a small aliquot of the aqueous nanoparticle suspension was placed onto a copper grid and dried at room temperature. Micrographs were obtained using a Tecnai G2 20 TEM (Thermo Fisher Scientific, Hillsboro, OR, USA). Particle dimensions were measured from TEM images ($n > 100$ individual particles) using ImageJ software (version 1.53t, National Institutes of Health, USA) to generate the size distribution.

Phase composition and crystal structure were assessed by X-ray diffraction (XRD) using a Bruker D2 Phaser diffractometer (Bruker, Karlsruhe, Germany). Samples were analyzed under Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$) at 30 kV and 10 mA. Diffractograms were collected from 20° to 80° (2θ) at 2° min^{-1} . Reflections were indexed against the standard ZnO wurtzite pattern (JCPDS Card No. 36-1451), and crystallite size was estimated using the Scherrer equation.

2.3. Bacterial Strain and Culture Conditions

An isolate of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was obtained from naturally infected rice seeds of the susceptible cultivar KDML105 collected from field-grown plants. The seeds were surface-sterilized and plated on peptone sucrose agar (PSA) medium to promote bacterial growth from the infected tissues. After incubation at 28°C for 72 h, yellow, mucoid colonies typical of *Xoo* developed around the infected seeds (Figure S1A). A single colony was subsequently purified by streak plating and maintained as the representative isolate for all experiments (Figure S1B). The isolate exhibited the characteristic biochemical reactions of *Xoo*, including a Gram-negative response, positive KOH string test, and starch hydrolysis. Pathogenicity of the isolate was confirmed using the leaf-clipping inoculation method on rice plants (*Oryza* 'KDML105'), following the procedure of Chumpol et al. [33]. The isolated bacterium was maintained in peptone sucrose broth (PSB) at 28°C for subsequent antibacterial assays.

2.4. Antibacterial Activity

2.4.1. Minimum Inhibitory Concentration (MIC) Assay

The minimum inhibitory concentration (MIC) represents the lowest dose of an antimicrobial that prevents any visible bacterial proliferation under standardized conditions. This value represents a bacteriostatic threshold rather than a bactericidal endpoint, indicating that bacterial proliferation is suppressed, although a minor fraction of cells may remain metabolically active [34].

The antibacterial activity of the commercial ZnO NPs against *Xoo* was evaluated by a broth microdilution method following the general principles of CLSI guidelines [35] and incorporating a resazurin-based viability indicator [36]. A primary suspension of ZnO NPs ($1024 \mu\text{g/mL}$) was prepared in sterile deionized water and subsequently diluted stepwise (twofold series) in peptone sucrose broth to yield final concentrations of 1–512 $\mu\text{g/mL}$ in 96-well plates. Each well received 100 μL of bacterial inoculum adjusted to approximately $1 \times 10^5 \text{ CFU/mL}$. Streptomycin sulfate (0.25–64 $\mu\text{g/mL}$) served as the positive control, and uninoculated wells containing only medium acted as blanks. Plates were incubated at 28°C for 48 h in darkness. Afterward, 20 μL of a 0.2% (w/v) resazurin solution (Sigma-Aldrich, USA) was added, and the plates were left for a further 2 h. Wells that turned pink or lost the blue coloration signified metabolic activity, whereas those remaining blue indicated growth inhibition. The MIC corresponded to the lowest nanoparticle concentration, showing no color change. All determinations were carried out in triplicate.

For mechanistic assays (Sections 2.4.3 and 2.4.4), bacterial cultures were subsequently exposed to ZnO NPs at $1\times$ MIC (the inhibitory concentration) and $2\times$ MIC (a lethal concentration above the inhibitory threshold) for 24 h. The 24 h period was selected to capture dynamic physiological responses, such as ROS generation and membrane damage, while avoiding complete bacterial death that typically occurs during 48 h MIC incubation. This design allowed for quantitative assessment of sublethal oxidative stress and cell integrity under controlled exposure conditions.

2.4.2. Agar Disc Diffusion Assay

The qualitative inhibition of *Xoo* by ZnO NPs was further examined using an agar diffusion approach adapted from the Kirby–Bauer protocol [37]. Peptone sucrose agar (PSA) plates were uniformly spread with actively growing *Xoo* cultures, adjusted to roughly 1×10^8 CFU/mL, using sterile cotton swabs. Circular sterile paper discs (6 mm in diameter) were impregnated with ZnO NP suspensions at 4, 8, 16, and 32 μ g per disc and carefully positioned on the inoculated agar. Discs containing sterile water served as negative controls, whereas those loaded with streptomycin (2 μ g/disc) acted as positive standards. Plates were incubated at 28 ± 2 °C for 48 h, after which the diameters of the clear zones surrounding each disc were measured with a digital caliper. The data provided a comparative validation of the antibacterial efficiency of ZnO NPs obtained in the broth-based MIC assays.

2.4.3. Live/Dead Fluorescence Assay

The membrane integrity of *Xoo* cells after exposure to ZnO NPs was examined using a fluorescence-based viability assay (LIVE/DEAD™ BacLight™, Invitrogen, Waltham, MA, USA). The method employs two nucleic acid dyes that distinguish viable from membrane-damaged cells through differential uptake. Actively growing bacterial cultures approximately 1×10^5 CFU/mL were incubated with commercial ZnO NPs at $1\times$ MIC (16 μ g/mL) and $2\times$ MIC (32 μ g/mL) for 24 h at 28 °C. Untreated samples served as negative controls, and cells treated with 70% isopropanol were used as positive (dead cell) references.

The 24 h exposure period was selected to allow evaluation of progressive cellular damage while maintaining sufficient metabolic activity for fluorescent detection. Extending incubation to 48 h, as used in the MIC assay, would have resulted in near-total growth inhibition and cellular disintegration, which prevents meaningful fluorescence visualization. Therefore, sublethal ($1\times$ MIC) and lethal ($2\times$ MIC) concentrations were chosen to represent early and advanced stages of membrane disruption, respectively.

After exposure, bacterial suspensions were centrifuged at $6000\times g$ for 5 min and rinsed twice with sterile phosphate-buffered saline (PBS) to remove unbound nanoparticles. The cell pellets were then resuspended in the working dye solution prepared from the LIVE/DEAD™ BacLight™ kit. The green-fluorescent SYTO9 label stains all cells by binding to nucleic acids, while the red-fluorescent propidium iodide (PI) penetrates only those with compromised membranes and competitively displaces SYTO9 from the DNA. Thus, intact cells appeared green (Ex/Em $\approx$ 480/500 nm), whereas membrane-damaged cells emitted red fluorescence (Ex/Em $\approx$ 490/635 nm). Fluorescence micrographs were captured using a Nikon ECLIPSE Ti2 inverted microscope (Nikon Instruments Inc., Tokyo, Japan) equipped with standard FITC and TRITC filter cubes (green channel: Ex = 480/25, Em = 525/30; red channel: Ex = 580/25, Em = 625/30), corresponding to the excitation–emission spectra of the respective dyes.

2.4.4. Intracellular ROS Detection

To evaluate oxidative stress as part of the antibacterial response, intracellular reactive oxygen species (ROS) levels in *Xoo* were assessed using the fluorescent probe DCFH-DA

(2',7'-dichlorodihydrofluorescein diacetate; Sigma-Aldrich, USA). Actively growing bacterial cultures (approximately 1×10^5 CFU/mL) were exposed to ZnO NPs at $1 \times$ MIC (16 $\mu\text{g/mL}$) and $2 \times$ MIC (32 $\mu\text{g/mL}$) for 24 h at 28 °C. The 24 h exposure was intentionally chosen to capture transient ROS generation before substantial cell death occurred, as prolonged incubation for 48 h (as in MIC determination) often leads to metabolic inactivation and a consequent loss of measurable fluorescence from ROS-dependent oxidation.

After nanoparticle exposure, the bacterial suspensions were pelleted by centrifugation at $6000 \times g$ for 5 min, gently rinsed twice with phosphate-buffered saline (PBS), and then incubated in a 10 μM DCFH-DA working solution for 20 min at 28 °C under dark conditions. Within viable cells, the non-fluorescent probe is enzymatically deacetylated to DCFH, which is subsequently converted to its fluorescent form, dichlorofluorescein (DCF), through oxidation by ROS. The resulting green fluorescence intensity (Ex/Em $\approx$ 488/525 nm) was visualized under a Nikon ECLIPSE Ni-U fluorescence microscope equipped with a FITC filter set. Autofluorescence and unstained samples were included to confirm the specificity of fluorescence signals.

2.4.5. Ultrastructural Analysis by TEM

To examine morphological alterations of *Xoo* cells following nanoparticle exposure, TEM was used as a high-resolution imaging approach. Exponentially growing bacterial cultures (approximately 1×10^5 CFU/mL) were treated with commercial ZnO NPs at $1 \times$ MIC (16 $\mu\text{g/mL}$) and $2 \times$ MIC (32 $\mu\text{g/mL}$) for 24 h at 28 °C. Untreated cells were processed in parallel as controls to provide baseline morphology for comparison. The 24 h period corresponded to the active phase of cellular stress, allowing for direct observation of nanoparticle–cell interactions consistent with the Live/Dead and ROS analyses.

Following incubation, bacterial pellets were obtained by centrifugation and washed twice with phosphate-buffered saline (PBS). Cell fixation was carried out overnight at 4 °C using 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), followed by secondary fixation with 1% osmium tetroxide for 1 h at room temperature. Dehydration was carried out using a graded ethanol series, followed by infiltration with epoxy resin, polymerization, and ultrathin sectioning as outlined in a previous report [5]. Imaging was performed using a Hitachi HT7700 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan) operated at 80 kV. Representative micrographs were analyzed for cell-wall deformation, intracellular nanoparticle uptake, and membrane disruption attributed to ZnO NP exposure.

2.4.6. Zn^{2+} Ion Release by ICP-OES

The concentration of Zn^{2+} ions released from commercial ZnO NPs was measured using inductively coupled plasma–optical emission spectroscopy (ICP-OES). Nanoparticle suspensions were freshly prepared in sterile deionized water at $1 \times$ MIC (16 $\mu\text{g/mL}$) and $2 \times$ MIC (32 $\mu\text{g/mL}$), and then gently agitated (150 rpm) at 28 °C for 24 h to simulate aqueous conditions comparable to antibacterial assays and to capture early ion-release behavior prior to aggregation.

After incubation, suspensions were centrifuged at $12,000 \times g$ for 15 min, and the resulting supernatants were passed through 0.22 μm syringe filters (Millipore, Billerica, MA, USA) to remove remaining particulates. Filtrates (5 mL) were acidified with 5% (v/v) ultrapure nitric acid (HNO_3 ; Reagecon Diagnostics Ltd., Shannon, County Clare, Ireland) and digested in a water bath at 95 ± 5 °C until clear. The cooled digests were adjusted to a final volume of 25 mL with deionized water and analyzed using a PerkinElmer Optima 8000 ICP-OES system (Waltham, MA, USA). Calibration standards (0.005–5.0 mg L^{-1}) and spiked blanks were run to verify analytical precision. Sterile water treated identically

without nanoparticles served as the negative control. The analysis was performed in accordance with APHA guidelines (APHA, AWWA, WEF, 2023; Sections 3120 B and 3030 E) [38].

2.5. Cytotoxicity Assay in Human Dermal Fibroblast (HDF) Cell Line

Human dermal fibroblasts (HDFs; ATCC[®] PCS-201-012[™]) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Waltham, MA, USA) containing 10% fetal bovine serum and 1% penicillin–streptomycin under standard conditions (37 °C, 5% CO₂). Cells at approximately 80% confluence were detached using TrypLE[™] Express (Gibco; Cat# 12604021), counted, and seeded into 96-well plates (1 × 10⁴ cells/well). After 24 h of attachment, the cells were treated with various concentrations of ZnO NPs (8–1024 µg/mL) for 24 h. Cell viability was assessed using the MTT colorimetric method as described previously [39]. Absorbance was measured at 570 nm with a standard microplate reader (Molecular Devices SpectraMax M5, San Jose, CA, USA). Relative viability (%) was determined against untreated controls. All experiments were performed in triplicate, and data are expressed as mean ± standard deviation (SD).

2.6. Statistical Analysis

Statistical evaluation of antibacterial, cytotoxicity, and Zn²⁺ ion release results was performed through one-way analysis of variance (ANOVA) with Duncan's post hoc comparison. Results are presented as mean values with their corresponding standard deviations (SD). Statistical significance was accepted at $p < 0.05$. All computations were conducted using SPSS version 22.0 (IBM, Armonk, NY, USA).

3. Results and Discussion

3.1. Physicochemical Characterization of Commercial ZnO NPs

The commercial ZnO nanoparticles (ZnO NPs) used in this study were obtained from Sigma-Aldrich (Product No. 544906). According to the supplier's specifications, these NPs are nanopowder with an average particle size of ~71 nm, a surface area of 15 m²/g, and a purity of ~79.4% Zn. However, the manufacturer does not disclose the synthesis method.

In general, large-scale ZnO nanopowders are typically produced through chemical or mechanochemical routes such as precipitation, sol–gel, hydrothermal, or high-energy ball-milling methods, which enable reproducible and cost-effective industrial-scale production [22–24,40,41]. In contrast, biosynthetic or green synthesis approaches, which employ reducing and stabilizing agents derived from plants or microorganisms, are mostly limited to laboratory-scale research and remain impractical for commercial manufacturing [13].

Given the industrial origin and large-scale production of commercial ZnO nanopowders, their physicochemical characteristics may vary across batches depending on precursor purity, synthesis temperature, and post-processing steps. Therefore, comprehensive characterization was necessary in this study to confirm the reproducibility and suitability of the commercial ZnO NPs prior to biological evaluation.

3.1.1. Morphology and Particle Size (FE-SEM and TEM Analysis)

FE-SEM images revealed that the commercial ZnO NPs exhibited irregular polyhedral morphologies, including hexagonal and short rod-like particles, distributed across the nanoscale field of view (Figure 1A). TEM analysis confirmed this heterogeneous morphology and showed well-dispersed nanoparticles with minimal agglomeration (Figure 1B). Particle size measurements based on more than 100 particles using ImageJ indicated a poly-disperse distribution ranging from 20 to 120 nm, with an average diameter of approximately 33 nm (Figure 1C).

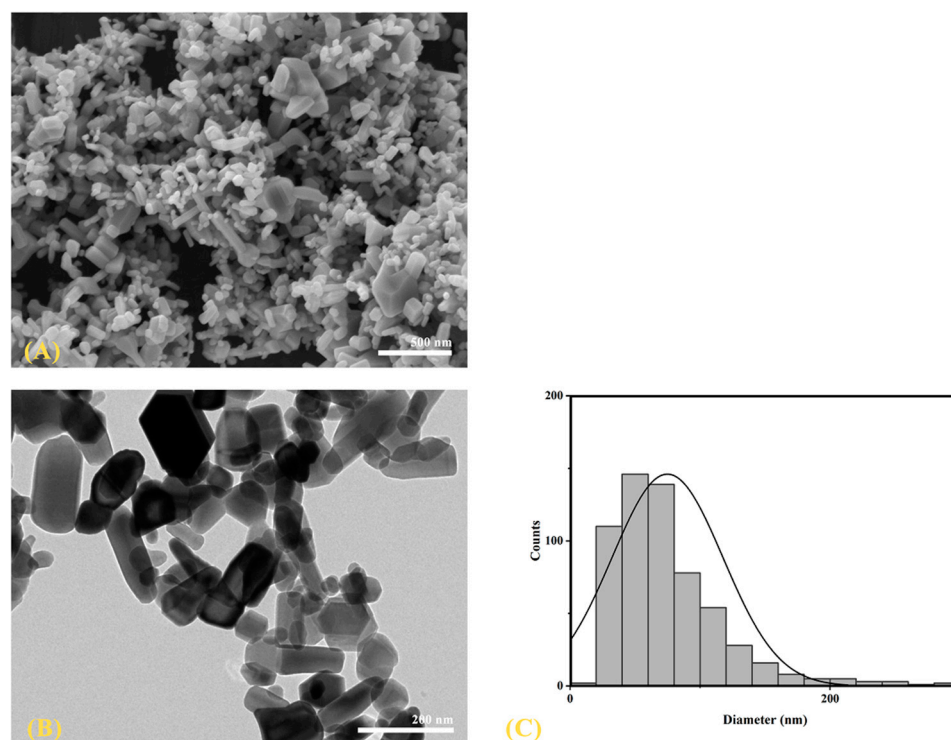


Figure 1. Morphological characterization of commercial ZnO NPs: (A) FE-SEM micrograph showing irregular polyhedral to hexagonal short-rod-like ZnO NPs with discrete nanoscale features; (B) TEM image confirming well-dispersed nanoparticles exhibiting mixed polyhedral and hexagonal morphologies with minimal aggregation; (C) particle-size distribution histogram derived from TEM measurements ($n > 100$), indicating a polydisperse range of 20–120 nm with an average diameter of approximately 33 nm.

Interestingly, the morphology and particle size observed in this study showed slight deviations from those reported in previous works using the same commercial product (Sigma-Aldrich, Cat. No. 544906) [25,26]. Konor et al. [25] described predominantly quasi-spherical ZnO NPs with a mean diameter of 45 nm, whereas Orozco-Messana et al. [26] reported more densely aggregated, nearly equiaxial crystallites of approximately 75 nm with a surface area of $8.9 \text{ m}^2 \text{ g}^{-1}$. In contrast, the present batch displayed more discrete polyhedral and short-rod morphologies with a smaller mean size ($\sim 33 \text{ nm}$). Such differences among studies employing the same catalog product likely reflect batch-to-batch variability or differences in sample preparation (e.g., dispersion method, drying conditions, or imaging parameters), all of which can influence the apparent particle dimensions and aggregation behavior.

Despite these variations, all results consistently confirmed the nanoscale dimensions and the presence of the hexagonal wurtzite phase that is typical of ZnO. The observed morphological heterogeneity and broad size distribution are characteristic of chemically synthesized ZnO NPs produced by sol-gel, precipitation, or hydrothermal processes that generally lack natural biomolecular capping agents to control crystal growth [41,42]. In such chemical routes, synthetic surfactants such as polyvinylpyrrolidone (PVP), cetyltrimethylammonium bromide (CTAB), or polyethylene glycol (PEG) are often used to minimize agglomeration [40,43], but incomplete removal or uneven adsorption of these surfactants can result in irregular shapes [42]. Chemical synthesis pathways are known to yield various ZnO morphologies, including rod-like [44–46], hexagonal [46,47], flower-like [46,48,49], honeycomb [50], pyramid [51], and thorn-like structures [52], depending on the type of precursor, surfactant, temperature, and reaction time [53]. Therefore, the mixture of polyhedral and short-rod structures observed in this batch is consistent with the morphological

diversity commonly associated with chemical synthesis processes, suggesting that the commercial ZnO NPs analyzed in this study were most likely derived from a conventional chemical synthesis route used for industrial-scale production.

3.1.2. Elemental Composition Analysis by EDX

EDX spectroscopy confirmed that zinc and oxygen were the predominant elements in the commercial ZnO NPs, accounting for 74.19 wt% Zn and 21.67 wt% O, with a minor carbon content of 4.14 wt% (Figure 2A; Table 1). The carbon signal was attributed to the conductive carbon tape used for sample mounting or possible surface adsorption of atmospheric CO₂. The measured Zn/O ratio was close to the theoretical stoichiometry of ZnO, indicating high elemental purity and supporting the crystallographic results obtained from XRD analysis.

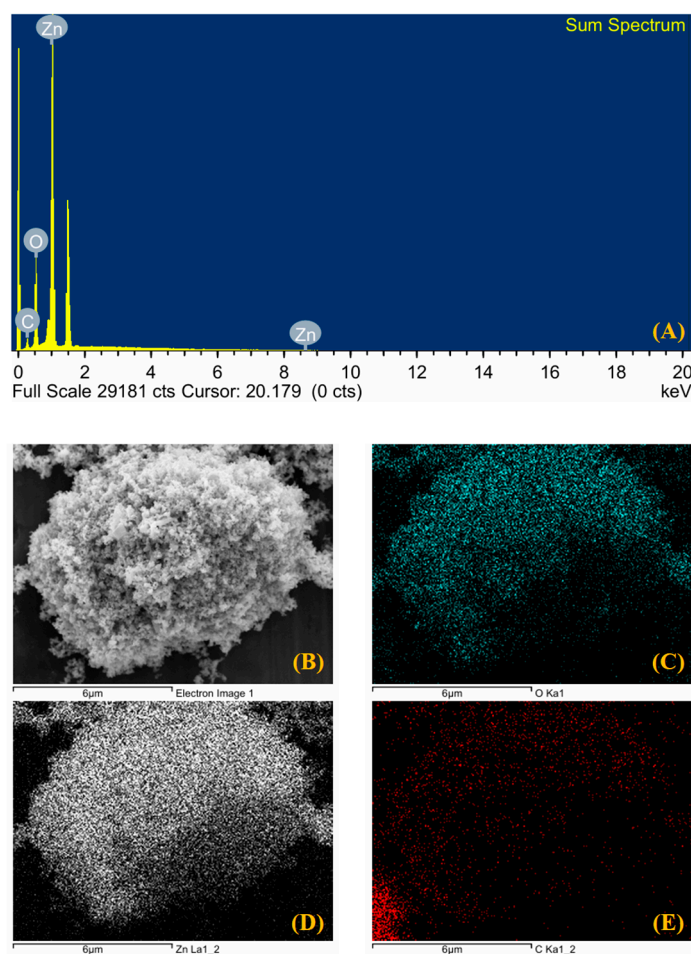


Figure 2. EDX and elemental mapping analysis of commercial ZnO nanoparticles: (A) EDX spectrum showing strong Zn and O peaks with a minor C signal attributed to the carbon substrate; (B) FE-SEM image of aggregated ZnO nanoparticles; (C) elemental map of oxygen (O-K α_1 , cyan); (D) elemental map of zinc (Zn-L α_1 , white); (E) elemental map of carbon (C-K α_1 , red).

Elemental mapping further demonstrated a homogeneous spatial distribution of the constituent elements. The FE-SEM image (Figure 2B) shows the aggregated nanoparticle morphology used as the reference for mapping, while the oxygen map (Figure 2C; O-K α_1 , cyan) and zinc map (Figure 2D; Zn-L α_1 , white) reveal uniform co-localization of Zn and O signals throughout the aggregates, confirming the compositional homogeneity of the oxide phase. A faint carbon distribution (Figure 2E; C-K α_1 , red) was observed both in

the background and lightly over the nanoparticle clusters, which can be ascribed to the underlying carbon substrate or trace adsorption of environmental carbon species.

Table 1. Elemental composition of commercial ZnO nanoparticles determined by EDX analysis. Data were collected from five representative areas on the particle aggregates.

Spectrum	C (wt%)	O (wt%)	Zn (wt%)	Total (wt%)
1	3.46	20.19	76.35	100.00
2	3.07	20.59	76.34	100.00
3	3.57	21.64	74.78	100.00
4	4.57	22.21	73.22	100.00
5	6.01	23.73	70.26	100.00
Mean $\pm$ SD	4.14 $\pm$ 1.19	21.67 $\pm$ 1.40	74.19 $\pm$ 2.55	100.00

To ensure analytical consistency, EDX spectra were collected from five randomly selected areas (Table 1 and Figure S2). The results displayed highly consistent Zn/O ratios with low standard deviations, supporting the reproducibility of the measurement and indicating that the analyzed ZnO NPs possess high purity and uniform elemental composition. Comparable Zn/O dominance has also been reported in biosynthesized ZnO NPs, although additional elemental peaks (C, N, P, S, and Cl) are often observed due to residual biomolecules from plant extracts [54,55].

3.1.3. Crystal Structure by XRD Analysis

XRD analysis confirmed that the commercial ZnO NPs exhibited the characteristic diffraction pattern of the hexagonal wurtzite phase (JCPDS Card No. 36-1451) (Figure 3). Prominent peaks at $2\theta = 31.76^\circ$, 34.41° , and 36.24° correspond to the (100), (002), and (101) planes, respectively, while additional reflections at (102), (110), (103), (112), (200), (201), (004), and (202) indicate high crystallinity and phase purity. The average crystallite size, estimated using the Scherrer equation ($D = K\lambda/\beta\cos\theta$), was approximately 33 nm (Table 2), in close agreement with the TEM-derived mean particle size.

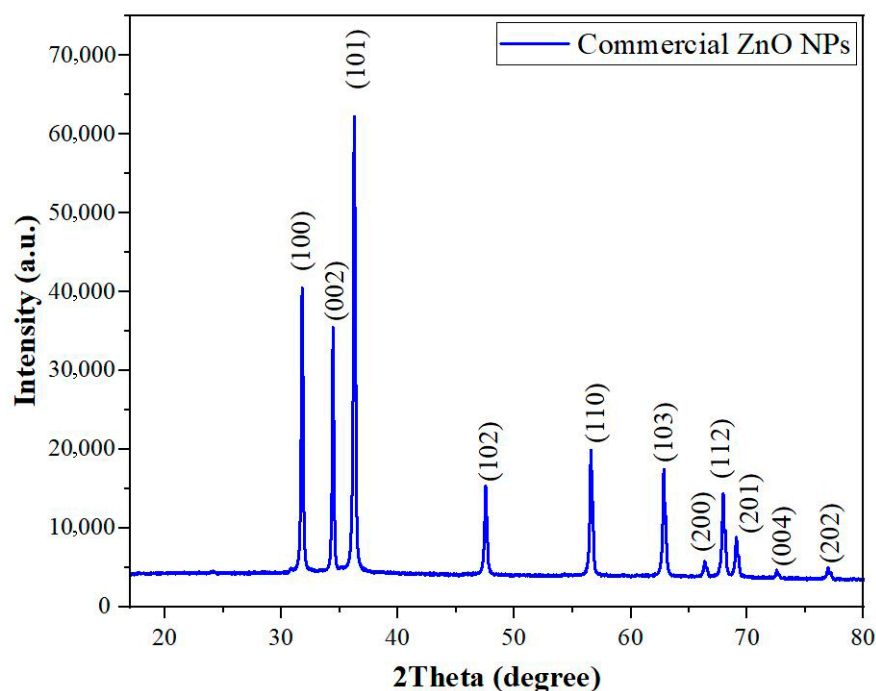


Figure 3. XRD pattern of commercial ZnO NPs showing sharp reflections characteristic of the hexagonal wurtzite phase (JCPDS No. 36-1451).

Table 2. Crystallite size (D) of commercial ZnO NPs calculated from the Scherrer equation.

Miller Indices (hkl)	2 θ (°)	FWHM (β , °)	D (nm)
(100)	31.76	0.2066	39.98
(002)	34.41	0.1827	45.52
(101)	36.24	0.2194	38.10
(102)	47.53	0.2534	34.26
(110)	56.59	0.2955	30.53
(103)	62.86	0.3170	29.37
(200)	66.38	0.3241	29.29
(112)	67.95	0.3293	28.89
(201)	69.09	0.3101	31.78
(004)	72.57	0.3110	31.78
(202)	76.97	0.4018	25.26
Average D (nm)			32.98

Comparable wurtzite-type diffraction features and crystallite dimensions have been reported for both chemically and green-synthesized ZnO nanoparticles, typically ranging from 30 to 50 nm [46,56,57]. Moreover, Orozco-Messana et al. [26] also reported a similar wurtzite pattern for the same commercial ZnO product (Sigma-Aldrich Cat. No. 544906), although their estimated crystallite size was larger (~75 nm), which may reflect batch-to-batch variability or post-synthetic treatment effects during industrial processing.

3.2. Antibacterial Activity of Commercial ZnO NPs Against *Xoo*

The antibacterial performance of the commercial ZnO NPs was assessed using broth microdilution and agar disc diffusion techniques. The MIC represents the lowest dose of an antimicrobial agent that halts visible microbial growth within the appropriate incubation period for each test organism [34]. In the case of *Xoo*, which shows relatively slow in vitro growth, the incubation was extended to 48 h to achieve complete inhibition.

The antibacterial activity of commercial ZnO NPs was assessed using broth microdilution and agar disc diffusion assays. In the broth microdilution assay with resazurin as an indicator, the MIC was determined to be 16 $\mu\text{g/mL}$, at which wells retained a blue color, indicating complete inhibition of bacterial metabolic activity. In contrast, wells in the untreated control turned pink, confirming active growth of *Xoo* (Figure 4A). This MIC value aligns with several reports of biosynthesized ZnO NPs tested against *Xoo*. For instance, ZnO NPs synthesized using *Matricaria chamomilla* (chamomile flower), *Olea europaea* (olive leaves), and *Solanum lycopersicum* (red tomato fruit) exhibited MICs of 16 $\mu\text{g/mL}$ [16]. Similarly, ZnO NPs derived from *Paenibacillus polymyxa* strain Sx3 displayed MIC values within the same range (16 $\mu\text{g/mL}$) [17]. In contrast, *Centella asiatica* (gotu kola) leaf extract-based ZnO NPs achieved a lower MIC of 8 $\mu\text{g/mL}$ against virulent *Xoo* strains [5], whereas larger particles synthesized from *Garcinia mangostana* (mangosteen peel) exhibited weak antibacterial effects (MIC up to 4000 $\mu\text{g/mL}$) [58]. Similarly, *Fusarium solani* (a filamentous fungus)-mediated ZnO NPs required 256–512 $\mu\text{g/mL}$ for inhibition [59].

The agar disc diffusion assay further confirmed the inhibitory effects of commercial ZnO NPs. At low concentrations (4 and 8 $\mu\text{g/disc}$), inhibition zones were negligible and not significantly different from the control. In contrast, clear and statistically significant inhibition zones were observed at 16 and 32 $\mu\text{g/disc}$ (Figure 4B; Table 3). These results align with previous studies that reported concentration-dependent inhibition of *Xoo* by ZnO NPs [5,15,16]. These parallels reinforce that the antibacterial potential of ZnO NPs against *Xoo* is robust across both commercial and biosynthesized sources, though biosynthesized forms may exhibit enhanced activity due to phytochemical stabilization [5].

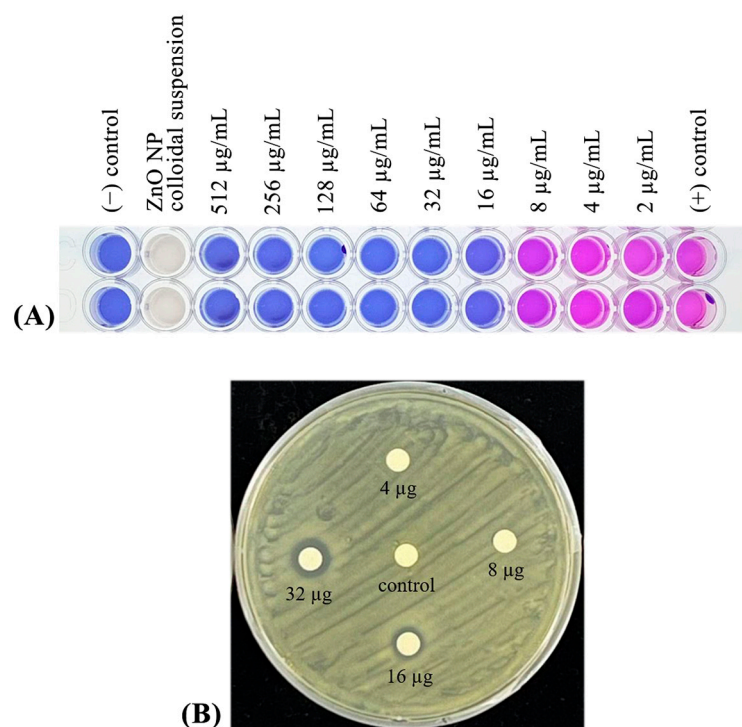


Figure 4. Antibacterial assays of commercial ZnO NPs against *Xoo*: (A) Broth microdilution test with resazurin indicator after 48 h at 28 °C showing MIC determination. The second well contained the ZnO NP colloidal suspension (1024 µg/mL in DI water), followed by twofold serial dilutions (512–2 µg/mL). Blue = growth inhibition; pink = bacterial activity. (–) control: medium only; (+) control: medium + *Xoo* without NPs. Each concentration was tested in duplicate across three independent 96-well plates. (B) Disc diffusion assay (48 h, 28 °C) showing dose-dependent inhibition zones for 4–32 µg/disc ZnO NPs; control disc = sterile DI water.

Table 3. Diameter of inhibition zones formed by *Xoo* after exposure to various concentrations of commercial ZnO NPs for 48 h at 28 °C. Data are presented as mean ± standard deviation (SD) based on three biological replicates (n = 3). Distinct lowercase letters indicate statistically significant differences among treatments according to Duncan’s test ($p < 0.05$).

ZnO NP Concentration (µg/disc)	Zone of Inhibition (mm)
0 (control)	0.00 ± 0.00 c
4	0.00 ± 0.00 c
8	0.00 ± 0.00 c
16	6.11 ± 0.29 b
32	7.84 ± 0.40 a

The size and morphology of ZnO NPs play a decisive role in their antibacterial performance, with several studies emphasizing their strong influence on efficacy, although conclusions remain inconsistent across reports [60–65]. Several studies demonstrated that spherical ZnO NPs show superior antibacterial performance compared with rod- and hexagonal-shaped particles, which has been attributed to their smaller average size, higher surface-to-volume ratio, and greater potential to generate ROS [61,65]. In contrast, other investigations reported that cuboidal or flower-like morphologies can display enhanced antibacterial activity relative to spherical particles, likely due to their higher density of surface defects or facet-dependent reactivity [62,63]. These contrasting results highlight that morphology cannot be considered in isolation; rather, size distribution, crystallinity, and surface chemistry collectively determine the antibacterial potency of ZnO NPs [64]. In the

present study, the commercial ZnO NPs exhibited mixed polyhedral shapes with an average size of ~30–40 nm, yet still achieved a low MIC of 16 µg/mL against *Xoo*. This suggests that even without strict morphological control, particle size within the nanoscale range and high crystallinity are sufficient to confer strong antibacterial activity, underscoring their utility as accessible nanobiocontrol agents.

3.3. Antibacterial Mechanism of Commercial ZnO NPs: Evidence from Membrane Integrity, ROS Generation, Ion Release, and Ultrastructural Investigations

To elucidate the antibacterial mechanism of commercial ZnO NPs, all mechanistic assays, including fluorescence-based viability, ROS detection, Zn²⁺ ion release, and TEM ultrastructural analysis, were conducted after 24 h exposure. This exposure period was specifically selected to capture early and intermediate physiological responses prior to complete bacterial death. Based on the MIC determination (48 h endpoint), bacterial growth is partially suppressed at 24 h but not yet fully eliminated, representing the phase when oxidative stress and membrane disruption become detectable yet quantifiable. Extending the incubation to 48 h would result in total cell lysis, thereby preventing meaningful assessment of intracellular ROS generation or membrane permeability changes. To further resolve the concentration-dependent response, two nanoparticle doses were examined, namely 1 × MIC (16 µg/mL) and 2 × MIC (32 µg/mL), allowing for differentiation between sublethal effects and complete bactericidal outcomes. The lower concentration represents the minimal inhibitory threshold that suppresses cell proliferation, whereas the higher concentration demonstrates the extent of irreversible damage associated with oxidative and structural collapse.

The Live/Dead BacLight assay employs two nucleic acid dyes with distinct membrane permeability properties. SYTO9 penetrates intact membranes and stains viable cells green, whereas propidium iodide (PI) enters only membrane-compromised cells and emits red fluorescence upon DNA intercalation [61]. In untreated *Xoo*, most cells exhibited bright green fluorescence with minimal red signals, indicating high viability. In contrast, treatment with 70% isopropanol, which served as a positive killing control, produced exclusively red-stained cells, confirming complete membrane disruption. After 24 h exposure to ZnO NPs, a concentration-dependent effect was evident. At 1 × MIC (16 µg/mL), cells displayed mixed green and red fluorescence, indicating partial membrane damage, while at 2 × MIC (32 µg/mL), most cells fluoresced red, reflecting extensive loss of membrane integrity (Figure 5A). These results suggest that membrane compromise is a downstream outcome following earlier oxidative stress events, rather than an immediate physical effect of nanoparticle contact.

Intracellular ROS production was examined using DCFH-DA, a widely used non-fluorescent probe for total ROS detection. Once inside the cells, DCFH-DA undergoes enzymatic deacetylation by intracellular esterases, yielding the non-fluorescent intermediate DCFH. This intermediate is then oxidized in the presence of ROS, such as superoxide, hydroxyl radicals, or hydrogen peroxide, resulting in the formation of the fluorescent molecule dichlorofluorescein (DCF), which emits green fluorescence at approximately 530 nm when excited at 485 nm [66]. In this study, autofluorescence controls (cells without dye) exhibited negligible signals (Figure 6A). Untreated controls stained with DCFH-DA showed only weak green fluorescence, reflecting basal ROS levels (Figure 6B). In contrast, ZnO NP treatment markedly enhanced intracellular ROS in a dose-dependent manner: cells exposed to 1 × MIC (16 µg/mL) displayed moderate fluorescence (Figure 6C), while those treated with 2 × MIC (32 µg/mL) exhibited brighter fluorescence signals (Figure 6D). The significant ROS accumulation observed within 24 h confirms that oxidative stress acts as an early trigger of the antibacterial response. Elevated ROS likely initiate lipid peroxidation,

enzyme inactivation, and nucleic acid oxidation, ultimately leading to loss of membrane integrity and cell death [11].

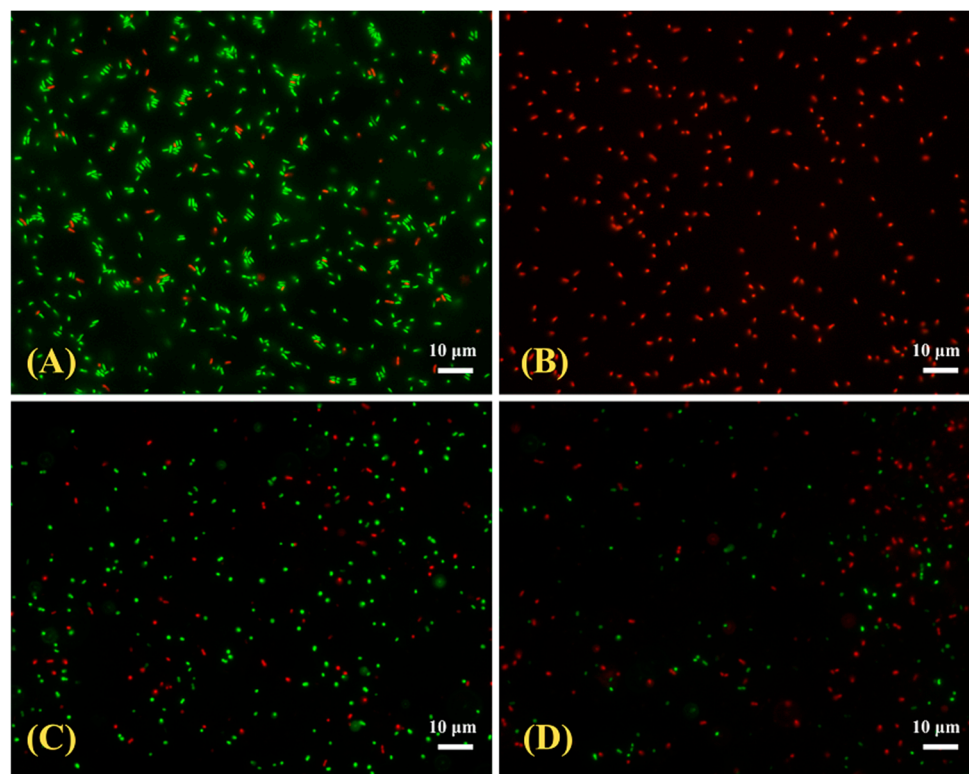


Figure 5. Fluorescence microscopy images showing membrane integrity of *Xoo* cells after treatment with commercial ZnO NPs, as determined by the Live/Dead BacLight assay: (A) untreated control showing mostly viable cells with intense green SYTO9 fluorescence and few red-stained (propidium iodide) cells, indicating intact membranes; (B) cells treated with 70% isopropanol (positive killing control) displaying exclusively red fluorescence, confirming complete membrane disruption; (C) cells treated with $1 \times$ MIC ($16 \mu\text{g/mL}$) ZnO NPs showing mixed green and red fluorescence, indicative of partial membrane damage; (D) cells treated with $2 \times$ MIC ($32 \mu\text{g/mL}$) ZnO NPs exhibiting predominantly red-stained cells, reflecting extensive membrane permeabilization. Scale bars = $10 \mu\text{m}$.

Zinc ion release from commercial ZnO NPs was quantified using ICP-OES after 24 h incubation in deionized (DI) water at 28°C (Figure 7). Detectable levels of Zn^{2+} were released from both $1 \times$ MIC ($16 \mu\text{g/mL}$) and $2 \times$ MIC ($32 \mu\text{g/mL}$) suspensions, whereas Zn^{2+} concentrations in DI water (control) remained below the detection limit. The concentration of dissolved Zn^{2+} increased significantly ($p < 0.05$) with nanoparticle dose, indicating partial dissolution of ZnO under the tested conditions. The higher Zn^{2+} levels at $2 \times$ MIC correspond with the stronger antibacterial responses observed in Live/Dead staining (Figure 5) and ROS fluorescence (Figure 6), suggesting that ion release contributes to the overall antibacterial activity. Previous reports have suggested that Zn^{2+} ions can enhance intracellular ROS formation and disrupt essential enzymatic systems, indicating that the antibacterial effect results from the combined influence of ionic and oxidative stress responses [64].

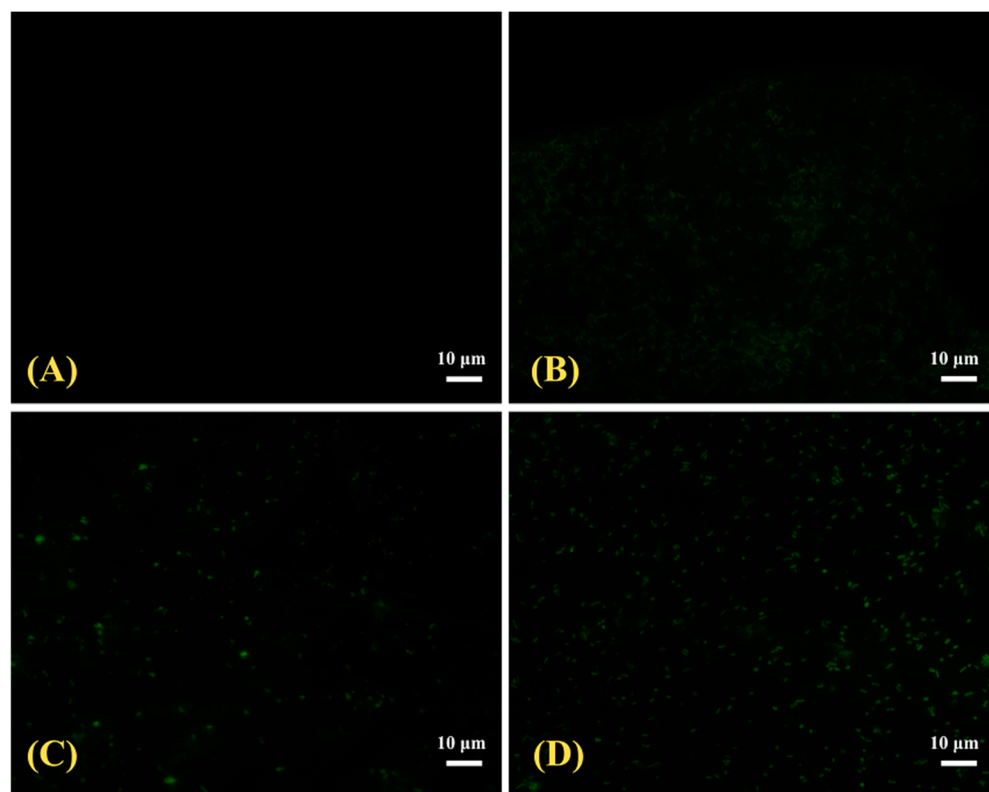


Figure 6. Fluorescence microscopy images showing intracellular ROS generation in *Xoo* cells after treatment with commercial ZnO NPs for 24 h, as visualized using DCFH-DA staining: (A) autofluorescence control (cells without DCFH-DA staining) showing no detectable signal; (B) untreated control exhibiting weak basal green fluorescence corresponding to normal ROS levels; (C) cells treated with $1 \times$ MIC ($16 \mu\text{g/mL}$) ZnO NPs displaying moderate fluorescence, indicating elevated ROS accumulation; (D) cells treated with $2 \times$ MIC ($32 \mu\text{g/mL}$) ZnO NPs showing intense green fluorescence, representing maximal ROS generation. Scale bars = $10 \mu\text{m}$.

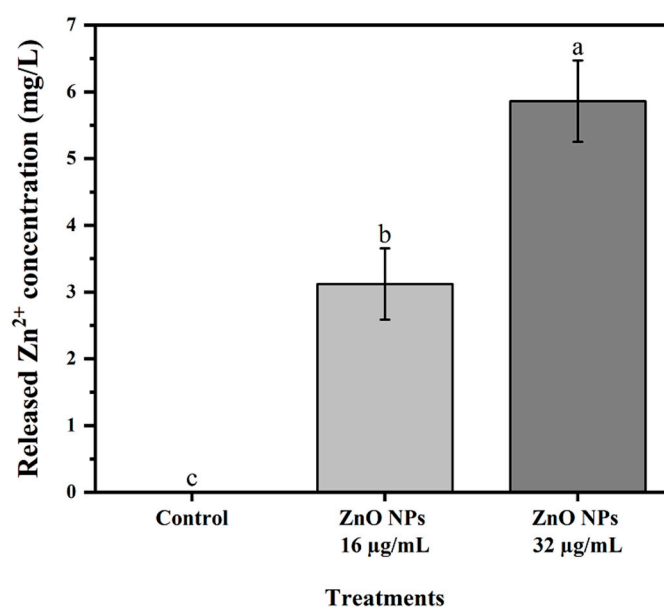


Figure 7. Zinc ion release from commercial ZnO NP suspensions determined by ICP-OES after 24 h incubation in deionized (DI) water at 28°C . Concentrations tested were 0 (control), 16, and $32 \mu\text{g/mL}$. Bars represent mean $\pm$ SD from three independent replicates, and different letters indicate statistically significant differences among treatments (Duncan's post hoc test, $p < 0.05$).

TEM micrographs revealed distinct morphological alterations in *Xoo* cells following exposure to commercial ZnO NPs (Figure 8). In untreated control cells (Figure 8A), the cell envelope appeared intact and smooth, with homogeneous cytoplasmic density. In contrast, ZnO NP-treated cells at $2\times$ MIC ($32\text{ }\mu\text{g/mL}$) (Figure 8B) exhibited disrupted membranes, irregular cell outlines, and cytoplasmic leakage. Notably, electron-dense nanoparticulate aggregates were observed adhering to and partially penetrating the cell envelope, suggesting direct contact and possible internalization of ZnO NPs into bacterial cells. This observation reinforces that while ROS and Zn^{2+} ions mediate intracellular biochemical stress, direct nanoparticle–membrane interaction contributes to mechanical disruption of the bacterial envelope.

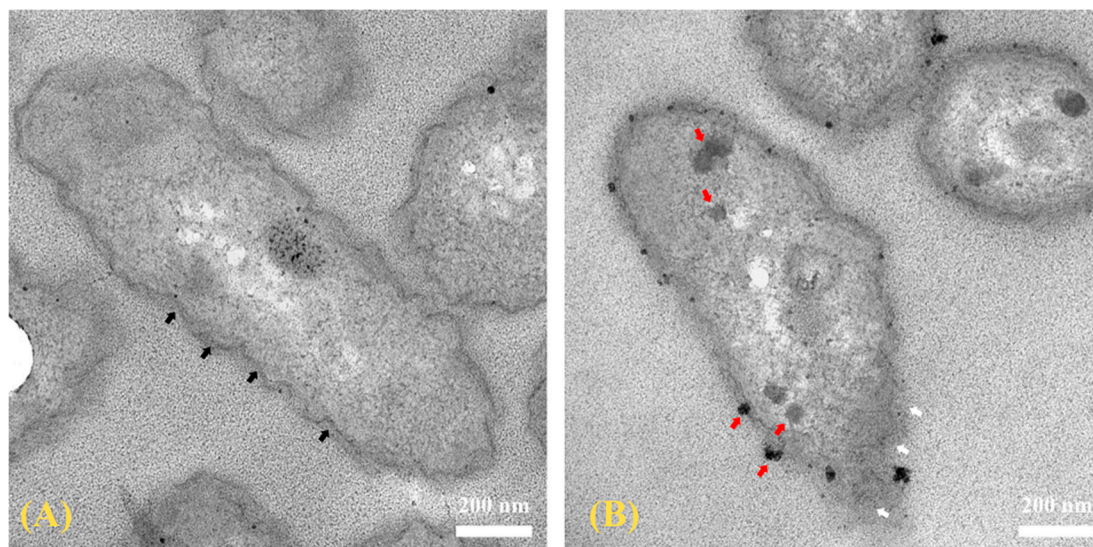


Figure 8. TEM images showing ultrastructural features of *Xoo* cells: (A) untreated control cells with intact membranes and uniform cytoplasmic content; (B) cells treated with $2\times$ MIC ($32\text{ }\mu\text{g/mL}$) commercial ZnO NPs exhibiting membrane rupture, cytoplasmic leakage, and nanoparticle adherence at the cell envelope. Black arrows indicate intact cell membranes, red arrows indicate ZnO nanoparticles adhered to the cell surface, and white arrows indicate membrane rupture sites. Scale bars = 200 nm.

At the physicochemical level, the morphology and size of ZnO NPs are also decisive factors influencing their antibacterial efficiency. The polyhedral and short rod-like morphologies observed in this study expose multiple reactive crystal facets that facilitate surface contact with bacterial membranes, thereby promoting ROS-mediated oxidation and localized Zn^{2+} ion exchange at the interface. Their 30–40 nm size range provides a high surface-to-volume ratio that enhances interfacial reactivity while maintaining colloidal stability. These morphology- and size-dependent attributes likely underlie the potent antibacterial performance of the tested ZnO NPs, consistent with earlier reports that particle geometry critically governs the balance between reactivity and biocompatibility [11].

Taken together, the combined evidence from fluorescence viability assays, ROS imaging, Zn^{2+} ion quantification, and TEM analyses consistently demonstrates that commercial ZnO NPs induce concentration-dependent cellular damage and oxidative stress in *Xoo*. Quantitative results revealed that both dissolved Zn^{2+} concentrations at $1\times$ and $2\times$ MIC and intracellular ROS fluorescence intensities increased proportionally with nanoparticle concentration, indicating that oxidative and ionic stresses act cooperatively to suppress bacterial proliferation.

Mechanistically, the antibacterial action of ZnO NPs can be explained through three interrelated processes previously established in the literature [5,11,64]. First, reactive oxygen species (ROS) such as hydroxyl radicals and superoxide anions oxidize cellular

macromolecules and initiate membrane destabilization [11]. Second, partial dissolution of ZnO releases Zn^{2+} ions, which interfere with enzymatic catalysis, nutrient transport, and metabolic homeostasis [64]. Third, direct nanoparticle contact with the bacterial envelope physically compromises membrane integrity, leading to cytoplasmic leakage and structural collapse [5,11].

In the present study, we provide experimental validation supporting all three mechanisms. DCFH-DA fluorescence imaging confirmed a dose-dependent elevation of intracellular ROS, particularly at $2\times$ MIC, corresponding to early oxidative damage. ICP-OES analysis verified measurable Zn^{2+} release after 24 h, indicating an additional ionic stress that disturbs metabolic equilibrium. Furthermore, Live/Dead staining and TEM micrographs revealed progressive membrane permeabilization, nanoparticle adherence at the cell surface, and extensive ultrastructural disruption.

Collectively, these complementary observations establish that commercial ZnO NPs inhibit *Xoo* via synergistic oxidative, ionic, and physical pathways, culminating in membrane rupture and cell death. The integrated mechanism is illustrated schematically in Figure 9, emphasizing how ZnO NPs concurrently trigger ROS generation, Zn^{2+} release, and envelope disruption to achieve potent antibacterial efficacy.

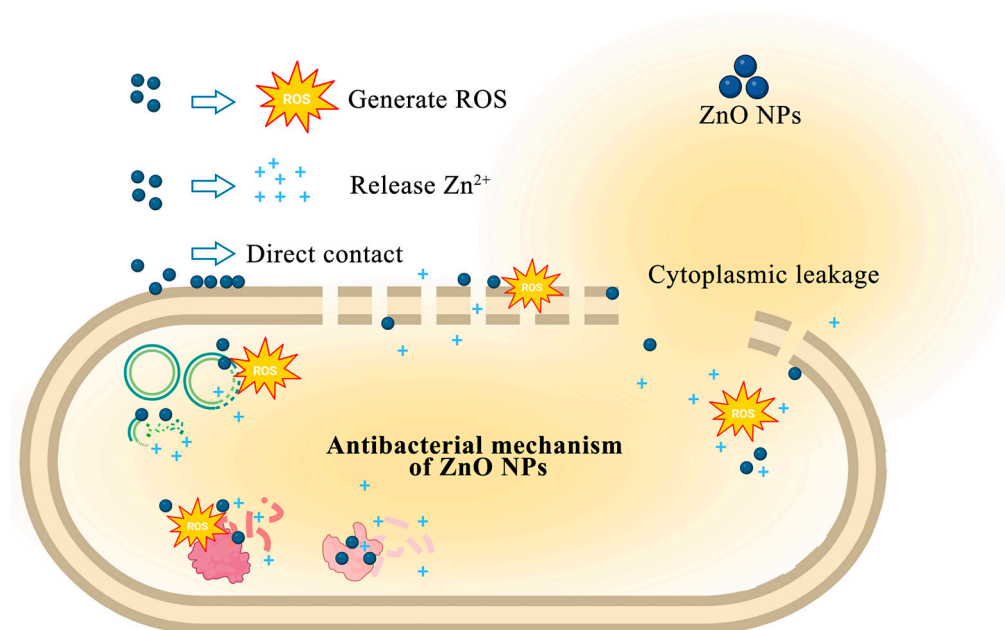


Figure 9. Proposed antibacterial mechanism of commercial ZnO NPs against *Xoo*. Schematic representation showing the three complementary modes of action: (i) ROS generation causing oxidative damage, (ii) Zn^{2+} ion release leading to ionic stress, and (iii) direct nanoparticle–cell contact resulting in membrane disruption and cytoplasmic leakage.

3.4. Cytotoxicity and Biocompatibility in Human Dermal Fibroblasts (HDF)

HDFs were employed as a representative mammalian cell model to assess the cytotoxic and biocompatible properties of commercial ZnO NPs. As dermal fibroblasts are directly involved in tissue repair and are among the first cell types to encounter nanoparticles through skin contact, they provide a physiologically relevant system for evaluating nanoparticle-induced cellular stress and viability [28,29]. Their pronounced sensitivity to oxidative and metal-induced stress further supports their use for nanotoxicological assessment [30,31]. Moreover, fibroblast-based studies have shown clear concentration- and time-dependent cytotoxicity in response to ZnO NP exposure, validating them as reliable models for toxicity evaluation [27].

The MTT colorimetric assay was employed as a standard *in vitro* method to quantitatively determine cell viability and to provide experimental evidence of the toxicity threshold in mammalian cells. The MTT assay in HDFs revealed a clear dose-dependent biphasic response to commercial ZnO nanoparticles. Cell viability remained near or above 100% between 4 and 256 $\mu\text{g/mL}$, followed by a decline at higher doses, reaching approximately 50% at 512 $\mu\text{g/mL}$ and 20% at 1024 $\mu\text{g/mL}$. The calculated IC_{50} was 433.6 $\mu\text{g/mL}$ (Figure 10), about 27-fold higher than the antibacterial MIC (16 $\mu\text{g/mL}$) against *Xoo*. This indicates a substantial safety margin between the concentration required to suppress the pathogen and the concentration at which measurable toxicity appears in mammalian cells.

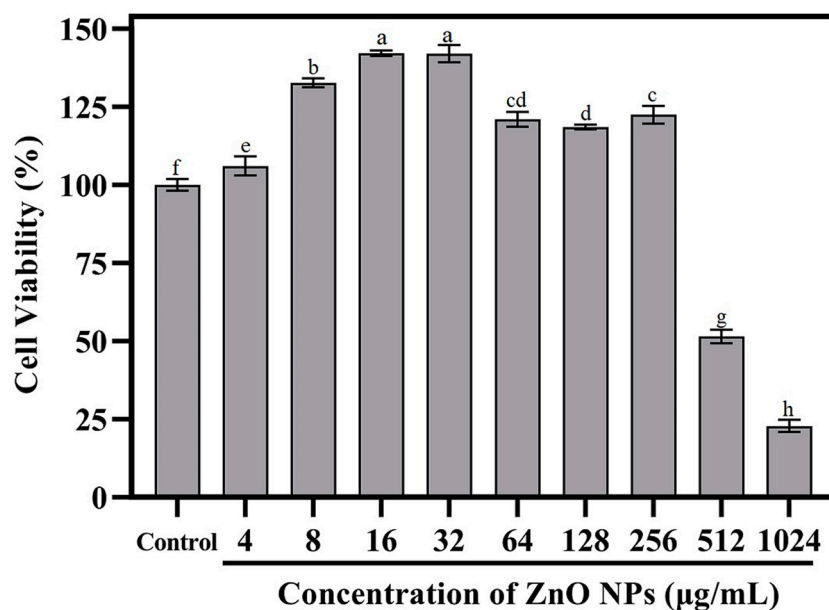


Figure 10. Cell viability of HDFs after 24 h exposure to ZnO NPs (4–1024 $\mu\text{g/mL}$). Data represent mean $\pm$ SD ($n = 3$). Different letters indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

Although the ZnO NPs used here share the same catalog number (Sigma-Aldrich, Cat. No. 544906) as those in several previous studies, the reported cytotoxicity and toxicity thresholds varied substantially, reflecting differences in both cell type and particle characteristics [25,67,68]. For instance, Hirano and Kanno [67] found strong autophagic and toxic responses in J774.1 macrophages, with LC3-II/p62 accumulation and ~90% cell death at 5 $\mu\text{g/mL}$ under serum-free conditions, whereas serum proteins markedly mitigated toxicity. Koner et al. [25] observed only moderate oxidative stress in primary fish hepatocytes, maintaining >80% viability at ≤ 10 $\mu\text{g/mL}$ after 72 h, while nitric oxide exerted a protective effect. In contrast, Colombo et al. [68] reported that irregularly shaped ZnO NPs (<100 nm) caused no significant alteration in transepithelial electrical resistance (TEER) or IL-6/IL-8 secretion in Caco-2 intestinal barriers, except at ≥ 50 –100 $\mu\text{g/mL}$ under basolateral exposure.

The discrepancies among these studies correspond well with variations in physico-chemical characteristics that directly influence nanoparticle toxicity. For instance, Orozco-Messana [26] described aggregated equiaxial ZnO particles (~75 nm crystallite size, $8.9 \text{ m}^2 \text{ g}^{-1}$ surface area), while Koner et al. [25] observed quasi-spherical particles (~45 nm). By contrast, the present ZnO NPs exhibited polyhedral and short rod-like morphologies averaging 30–40 nm. Such diversity likely reflects batch-to-batch differences in synthesis or post-processing, which affect surface area, Zn^{2+} ion release, and the resulting biological reactivity and toxicity. Previous reports have shown that the shape and size of ZnO NPs are

critical determinants of their toxic behavior [69,70]. Spherical ZnO NPs generally exhibit higher surface reactivity and faster Zn^{2+} ion dissolution, leading to stronger oxidative stress, whereas faceted or rod-like forms dissolve more slowly, thereby reducing toxicity and demonstrating better biocompatibility at sub-toxic levels [71].

Particle size and morphology are therefore key factors defining both antibacterial activity and cellular toxicity. Smaller ZnO NPs, though more antibacterial, tend to cause greater oxidative stress in mammalian cells due to rapid ion dissolution [69]. Conversely, mesoporous or biologically capped particles reduce toxicity by stabilizing Zn^{2+} release [72,73]. The moderate size (30–40 nm) and faceted morphology of the present commercial ZnO NPs likely contributed to their favorable balance between antibacterial efficacy and reduced cytotoxicity, as reflected by the high HDF viability (>80%) at concentrations up to 256 $\mu\text{g/mL}$. Moreover, consistent with previous fibroblast-based assays, ZnO NP-induced cytotoxicity generally follows both dose- and time-dependent trends, as demonstrated in human periodontal ligament and mouse dermal fibroblasts [27]. This observation reinforces that fibroblast responses to ZnO exposure are governed not only by concentration but also by exposure duration, reflecting their high sensitivity to oxidative and ionic stress. Furthermore, the biphasic response pattern observed here, characterized by enhanced cell proliferation at low to moderate concentrations and toxicity at higher doses, is consistent with earlier biomedical findings. At sub-cytotoxic levels, ZnO NPs can promote fibroblast proliferation and collagen synthesis, whereas excessive exposure induces oxidative stress and apoptosis through ROS-mediated mechanisms [32,74].

Taken together, these findings provide direct experimental and mechanistic evidence of ZnO NP toxicity thresholds in mammalian cells. They also indicate that the tested commercial ZnO NPs achieve an optimal balance between antibacterial activity and mammalian safety. In practical crop protection scenarios such as foliar spray or seed coating, ZnO NPs are typically applied at doses intended to contact the pathogen locally rather than to expose mammalian cells systemically, suggesting that effective antibacterial levels can be achieved without approaching the toxic range observed in vitro. Nonetheless, confirming these safety margins under agronomically relevant conditions remains essential, since nanoparticle behavior in complex environments (for example soil–plant–microbe systems and aquatic runoff) may influence exposure, persistence, and ecological risk.

4. Conclusions

This study provides the first integrated evaluation of commercially available ZnO NPs against the rice bacterial blight pathogen *Xoo* and in mammalian cells. The nanoparticles exhibited strong antibacterial activity with an MIC of 16 $\mu\text{g/mL}$ and a clear dose-dependent response. Mechanistic investigations indicated that bactericidal effects were associated with ROS formation, Zn^{2+} ion release, and membrane disruption, as verified through fluorescence imaging, ICP-OES analysis, and TEM observations. Human dermal fibroblasts showed high tolerance, with no cytotoxicity up to 256 $\mu\text{g/mL}$ and reduced viability only at 512 $\mu\text{g/mL}$, confirming a wide safety margin between antibacterial efficacy and mammalian cell compatibility. Although these nanoparticles shared the same catalog number as those reported elsewhere, they displayed distinct morphological and crystallographic features, underscoring the importance of verifying physicochemical characteristics such as particle size and shape even for standardized commercial nanopowders.

Given that effective antibacterial concentrations were far below cytotoxic thresholds observed in vitro, the findings suggest minimal risk to non-target mammalian cells under agricultural exposure conditions. However, as all assays were conducted under static in vitro systems, future investigations should extend to soil-based or dynamic plant–microbe environments to better reflect real-world interactions. Moreover, while fibroblast

assays provide an initial indication of biocompatibility, further in vivo validation using animal model systems is recommended to assess systemic and environmental biosafety. Overall, the results highlight the potential of commercial ZnO NPs as practical and sustainable nanobiocontrol agents for managing bacterial leaf blight in rice.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/applnano6040026/s1>, Figure S1: Isolation and purification of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) from naturally infected rice seeds (*Oryza* ‘KDML105’). (A) Growth of *Xoo* colonies emerging from infected rice seeds plated on peptone sucrose agar (PSA) after 72 h incubation at 28 °C. (B) Purified yellow, mucoid colonies of *Xoo* obtained by streak plating on PSA medium; Figure S2: EDX spectra of commercial ZnO NPs recorded from five representative areas confirming Zn and O elemental composition and sample homogeneity.

Author Contributions: Conceptualization, W.M.; methodology, T.J., P.R. and K.R.; software, T.J. and P.R.; validation, T.J., P.R., K.R. and W.M.; formal analysis, T.J. and W.M.; investigation, T.J., P.R., K.R., D.M. and W.M.; resources, D.M. and W.M.; data curation, T.J., P.R. and K.R.; writing—original draft preparation, T.J. and W.M.; writing—review and editing, T.J., P.R., D.M. and W.M.; visualization, T.J., P.R. and K.R.; supervision, W.M.; project administration, W.M.; funding acquisition, D.M. and W.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Khon Kaen University Research Program Funding (grant number RP66-1-003); Suranaree University of Technology (SUT), Thailand Science Research and Innovation (TSRI), and the National Science, Research and Innovation Fund (NSRF) (grant number 195243); and the National Research Council of Thailand (NRCT) (project number NRCT813/2563).

Data Availability Statement: The data presented in this study are available in this article and the Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Acknowledgments: The authors would like to express their sincere gratitude to the Research Instrument Center, Khon Kaen University (RIC-KKU), for providing analytical facilities and technical assistance. Special thanks are extended to Kunthaya Ratchaphonsaenwong of the Research and Academic Services Division, Faculty of Science, Khon Kaen University, for her technical assistance in TEM imaging, and to Ornuma Kalawa and Choulong Veann from the School of Physics, Institute of Science, Suranaree University of Technology (SUT), for their valuable support in SEM and XRD analyses. The authors also appreciate the Faculty of Science, Khon Kaen University, for providing research facilities and institutional support throughout this study.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

BLB	Bacterial Leaf Blight
DCFH-DA	2',7'-Dichlorodihydrofluorescein Diacetate
EDX	Energy-Dispersive X-ray Spectroscopy
FE-SEM	Field-Emission Scanning Electron Microscopy
HDF	Human Dermal Fibroblasts
ICP-OES	Inductively Coupled Plasma–Optical Emission Spectroscopy
MIC	Minimum Inhibitory Concentration
PI	Propidium Iodide
ROS	Reactive Oxygen Species

TEM	Transmission Electron Microscopy
Xoo	Xanthomonas oryzae pv. oryzae
XRD	X-ray Diffraction
ZnO NPs	Zinc Oxide Nanoparticles

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