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BIOSYNTHESIS OF ZNO NANOPARTICLES USING CELL-FREE SUPERNATANT OF METHYLOBACTERIUM RADIOTOLERANS: CHARACTERIZATION AND EVALUATION OF THEIR NANOPRIMING POTENTIAL IN GREENGRAM

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ABSTRACT
The present study investigated the biosynthesis of zinc oxide nanoparticles (ZnO NPs) using the cell-free supernatant of Methylobacterium radiotolerans and evaluated their potential as seed nanopriming agents in greengram (Vigna radiata L.) cv. CO 9. The biosynthesized nanoparticles were characterized using Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), field emission scanning electron microscopy (FESEM) and energy-dispersive X-ray spectroscopy (EDX). The nanopriming potential was assessed at concentrations of 100, 200, 300, 400 and 500 ppm, alongside untreated and hydroprimed controls. Germination, seedling growth, vigour indices and biochemical parameters, including α-amylase, dehydrogenase, catalase and peroxidase activities, were evaluated. XRD analysis confirmed the hexagonal wurtzite crystalline structure of ZnO, with an average crystallite size of 30.4 nm. FTIR analysis indicated the presence of hydroxyl, amide and C–O functional groups, while FESEM revealed irregularly shaped, aggregated particles with dimensions an average particle size of 61.23 nm. EDX analysis confirmed the presence of zinc and oxygen as the predominant elements. Among the nanopriming treatments, 200 ppm ZnO NPs recorded the highest germination (96%), root length (17.12 cm), shoot length (20.43 cm) and seedling vigour indices I and II (3605 and 24, respectively). This treatment also recorded the highest activities of enzymes. Dry matter production showed no statistically significant treatment effect. The findings demonstrate the potential of M. radiotolerans-mediated ZnO nanoparticles as seed nanopriming agents for improving germination, seedling vigour and germination-associated biochemical activities in greengram.

KEYWORDS: Methylobacterium radiotolerans, zinc oxide nanoparticles, green synthesis, cell-free supernatant, nanopriming, Vigna radiata, seedling vigour.

INTRODUCTION
Nanotechnology has emerged as one of the most transformative disciplines of the 21st century, with metal oxide nanoparticles occupying a central position in materials science, catalysis, environmental remediation, and biomedical applications[1].Among the various metal oxides, zinc oxide (ZnO) has garnered exceptional research interest owing to its unique combination of semiconducting, piezoelectric, and optical properties, coupled with its biocompatibility, low toxicity, and economic viability[2][3]. ZnO is a wide-band-gap semiconductor with a large exciton binding energy of 60 meV, rendering it highly efficient for photocatalytic, optoelectronic, and antimicrobial applications [2] [4]. In the agricultural domain, ZnO nanoparticles have demonstrated remarkable potential as nano-fertilizers, seed priming agents, and elicitors of abiotic stress tolerance, primarily through the modulation of reactive oxygen species (ROS) homeostasis and enhancement of antioxidant enzyme activity[5] [6].
The conventional synthesis of ZnO nanoparticles relies on physical and chemical methods such as sol-gel processing, hydrothermal synthesis, chemical vapor deposition, and precipitation techniques [7]. While these approaches offer precise control over particle size and morphology, they are often associated with significant drawbacks, including high energy consumption, the use of toxic solvents and stabilizing agents, generation of hazardous by-products, and poor biocompatibility of the resulting nanomaterials[8][9]. In response to these

limitations, the paradigm of "green synthesis" has gained substantial momentum, leveraging biological entities including plants, algae, fungi, and bacteria as sustainable, eco-friendly nano-factories for the bottom-up fabrication of metal oxide nanoparticles[10].

Microbial-mediated synthesis represents a particularly promising avenue within the green synthesis framework. Bacteria, with their rapid growth rates, ease of cultivation, and remarkable metabolic diversity, can reduce metal ions and nucleate nanocrystals through extracellular or intracellular methods [11]. The cell wall and cytoplasm are sites of intracellular synthesis. The extracellular metabolites produced by bacteria including proteins, polysaccharides, organic acids, and redox-active enzymes serve dual roles as reducing agents and stabilizing capping layers, thereby controlling particle size, shape, and surface chemistry [12] [13]. More recently, cell-free extracts (CFEs)the biomolecule-rich supernatant obtained after removing bacterial cells have emerged as an efficient alternative to whole cell synthesis, offering simpler downstream purification, easier standardization, and reduced contamination from cellular debris while retaining the proteins, enzymes, and peptides responsible for nanoparticle reduction and capping[14]. Among the bacterial genera explored for nanoparticle synthesis, Gram-positive organisms such as Bacillus and Lactobacillus have been extensively studied, while the potential of Pink-Pigmented Facultative Methylotrophs (PPFMs) remains largely underexplored.

Methylobacterium radiotolerans is a Gram-negative, pink-pigmented, facultatively methylotrophic bacterium [15]. This organism is renowned for its metabolic versatility, radiation resistance, and ability to colonize the phyllosphere and rhizosphere of diverse plant species [16]. M. radiotolerans produces a distinctive array of extracellular metabolites, including carotenoid pigments, exopolysaccharides, and methanol-dehydrogenase enzymes, which confer unique redox and surface-active properties [15] [16]. Despite these advantageous characteristics, the application of Methylobacterium radiotolerans as a biological source for the synthesis of metal oxide nanoparticles remains insufficiently explored, representing a significant gap in the current knowledge landscape.

Seed priming, a pre-sowing hydration treatment, is a cheap and farmer-adoptable way to hasten germination and uniformity. Priming with nanoparticle suspensions, termed nanopriming, has been proposed as a further step that can improve water uptake through seed coat nanopore formation and deliver micronutrients or signalling cues directly to the embryo[17] [18]. Nanopriming, the application of nanoparticle suspensions as seed treatments prior to sowing has emerged as a cutting-edge strategy to enhance seed germination, seedling vigor, and stress tolerance in crop plants [19].

Greengram (mungbean, Vigna radiata L. Wilczek) is a short-duration pulse that supplies dietary protein and fixes atmospheric nitrogen, yet stand establishment is frequently poor under suboptimal soil moisture, salinity and micronutrient deficiency. Zinc is an essential micronutrient required for enzyme activation, chloroplast development and primary metabolism, and zinc deficiency is widespread in cropping soils [20]. In greengram specifically, ZnO NPs applied as seed treatments have been reported to modify germination and seedling growth in a concentration-dependent manner [21] and microwave-synthesized cysteine-capped ZnO NPs used as priming agents improved germination and growth under moisture stress, with zinc uptake in roots confirmed by EDAX and fluorescent labelling [22]

In light of these considerations, the present study was designed with the following objectives: (1) to develop a cell free supernatant mediated green synthesis protocol for ZnO nanoparticles using the pink-pigmented facultative methylotroph Methylobacterium radiotolerans; (2) to comprehensively characterize the structural, morphological and surface chemical properties of the biosynthesized ZnO using X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR),Field Emission Scanning Electron Microscopy (FESEM) and energy-dispersive X-ray spectroscopy (EDX) and (3) to evaluate their nanopriming efficacy on germination, seedling growth and physiological and biochemical traits of greengram.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

The pink-pigmented facultative methylotroph Methylobacterium radiotolerans (GenBank accession no. PV628277) was used as the biological medium for the cell-free supernatant-mediated synthesis of ZnO nanoparticles. The subculture was obtained from the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. The bacterial culture was maintained on glycerol peptone liquid medium at 29 ± 2 °C for 24–48 h.

Biosynthesis of ZnO Nanoparticles

Preparation of cell free supernatant

The biosynthesis of ZnO nanoparticles was carried out using a cell-free supernatant prepared from Methylobacterium radiotolerans. The grown bacterial culture was centrifuged at 6000 rpm for 15 min to remove bacterial cells and cell debris, and the resulting cell-free supernatant was collected and used for subsequent nanoparticle synthesis[23](Fig.1)

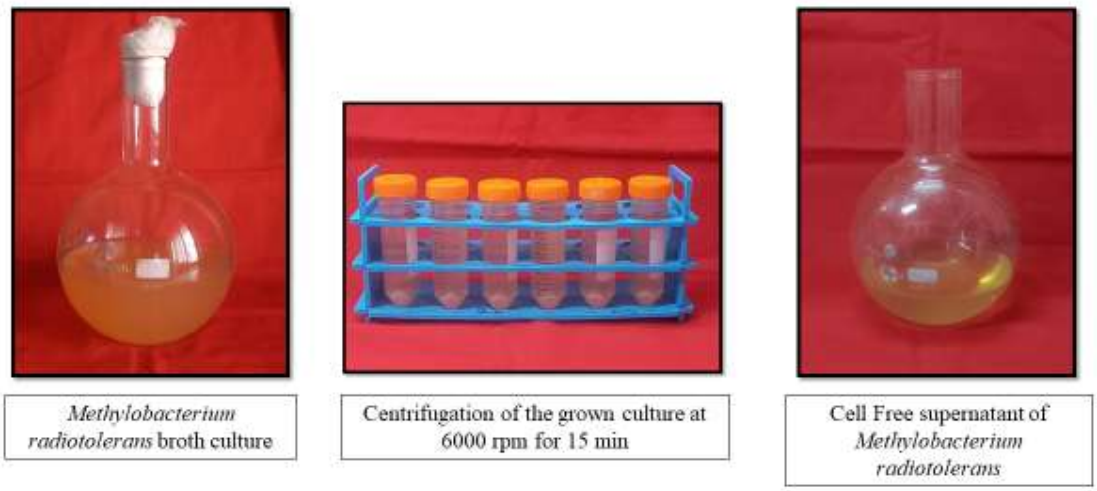


Fig. 1. Preparation of cell-free supernatant from *Methylobacterium radiotolerans*.

Procedure for synthesis of ZnO nanoparticle from Cell free Supernatant of Methylobacterium radiotolerans

The synthesis of ZnO nanoparticles was adapted from the procedure of Al Tameemi et al., (2023) [24] with slight modifications. Two hundred millilitres of 0.1 M $ZnSO_4$ solution was added dropwise from burette to 1 L of cell-free supernatant under constant magnetic stirring at 80 °C until a pale white precipitate appeared at the bottom of the flask (Fig 2.). The mixture was then incubated at room temperature for 24 h. The settled particles were harvested by centrifugation at 6000 rpm for 15 min. They were washed repeatedly with deionized water to remove unreacted precursor ions, residual proteins and cellular debris. The particles were then dried at 80 °C for 2 h in a hot-air oven, followed by calcination in a muffle furnace at 400 °C for 3 h. The dried powder was gently ground with a mortar and pestle and stored in an airtight container at room temperature for further characterization.

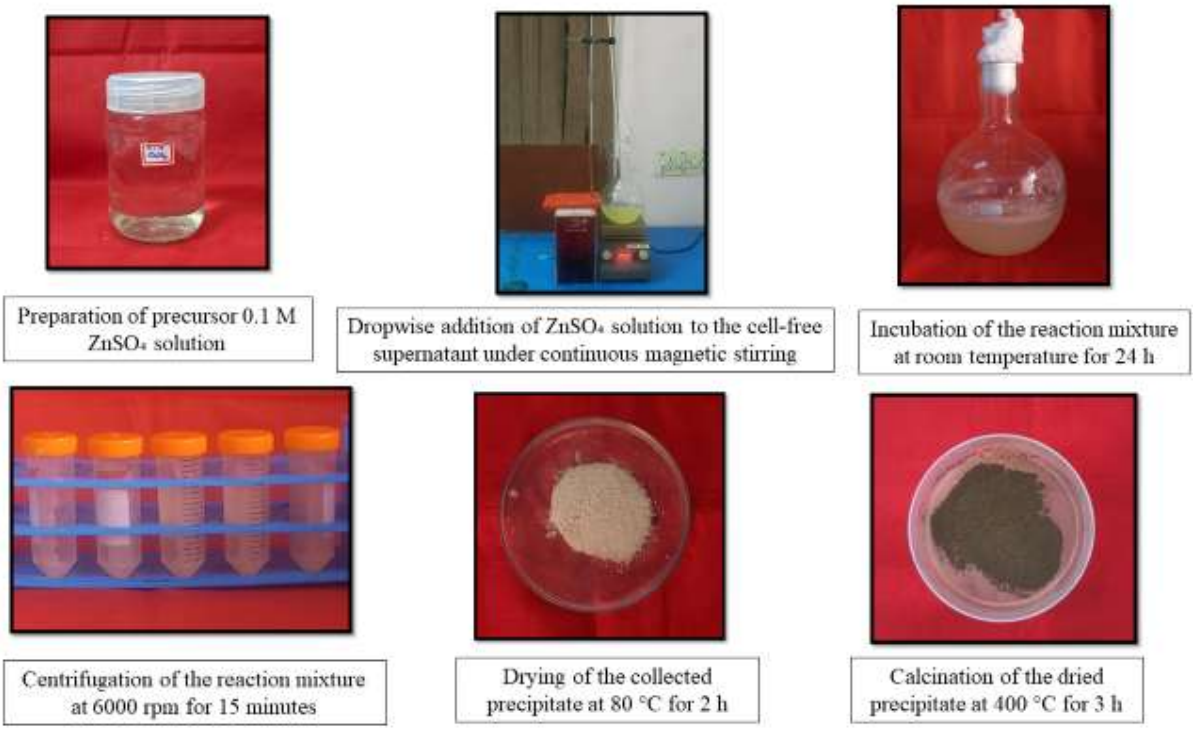


Fig. 2. Biosynthesis of ZnO nanoparticle using the cell-free supernatant of *Methylobacterium radiotolerans*

Characterization of Biosynthesized ZnO Nanoparticles

X-Ray Diffraction (XRD) Analysis

The crystalline phase and structural properties of the biosynthesized ZnO nanoparticles were determined by powder X-ray diffraction (XRD) using a PANalytical X'PERT-PRO diffractometer (PW3050/60 goniometer, Netherlands) available at Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore. The instrument is equipped with a Cu anode radiation source ($K\alpha_1$ = 1.54060 Å, $K\alpha_2$ = 1.54443 Å) operating at 45 kV and 30 mA. The diffraction pattern was recorded over a 2θ range of 10.00°–79.98° with a step size of 0.017° and a scan step time of 5.715 s. The crystallite size (D) was calculated from the full width at half maximum (FWHM) of the indexed diffraction peaks using the Scherrer equation proposed by Klug and Alexander, 1974 [25]

$D = K\lambda / (\beta \cos \theta)$

where K is the Scherrer constant (0.9), λ is the X-ray wavelength (1.5406 Å), β is the FWHM in radians, and θ is the Bragg diffraction angle.

Fourier-Transform Infrared (FTIR) Spectroscopy

The surface functional groups associated with the ZnO nanoparticles were analyzed by Fourier-transform infrared (FTIR) spectroscopy using an FT/IR-6800 type A spectrometer (JASCO Corporation, Japan) equipped with an ATR PRO ONE accessory with a 45° incident angle. The spectrum was recorded over the wavenumber range of 4000–349 cm^{-1} at a resolution of 4 cm^{-1} with 30 accumulations per scan. The characteristic vibrational modes were assigned by comparison with standard reference spectra.

Field Emission Scanning Electron Microscopy with Energy-Dispersive X-Ray Spectroscopy (FE-SEM-EDX)

The surface morphology, particle size, and shape of the biosynthesized ZnO nanoparticles were examined by field emission scanning electron microscopy (FE-SEM) using a MIRA3 high-performance FE-SEM (TESCAN, Czech Republic). The field emission electron source provides high-resolution imaging, enabling visualization of particle size, shape, and structural details at the nanometer scale under varying magnifications. For imaging, the dried nanoparticle powder was mounted on a carbon tape adhered to an aluminum stub and sputter-coated with a thin layer of gold to enhance electrical conductivity. Secondary electron images were captured at various magnifications to assess the morphology and agglomeration behavior of the nanoparticles.

EDX analysis was performed to determine the elemental composition and distribution of the biosynthesized nanoparticles using the integrated EDX detector with EDAX APEX software. The system detects X-rays emitted from the sample under the electron beam, confirming the elemental presence and determining the distribution in the sample. Quantitative details, including the atomic and weight percentages of each element, were obtained using SmartQuant software.

Nanopriming Treatment and Seed Germination Assay

Seed Source and Experimental Site

Seeds of greengram [Vigna radiata (L.) var. CO 9] were procured from the Department of Pulses, Tamil Nadu Agricultural University, Coimbatore. The study was carried out during 2025 at the Department of Seed Science and Technology, Tamil Nadu Agricultural University, Coimbatore. All chemicals used in this study were of analytical grade and acquired from Sigma-Aldrich, India.

Nanopriming of greengram seeds using ZnO nanoparticles

The biosynthesized ZnO nanoparticles were suspended in sterile distilled water at concentrations ranging from 100 to 500 ppm (1 ppm = 1 mg/L for aqueous suspensions) The treatment details are as follows: control dry seeds (T_0), hydropriming (T_1), 100 ppm (T_2), 200 ppm (T_3), 300 ppm (T_4), 400 ppm (T_5), 500 ppm (T_6).The suspensions were sonicated in ultrasonic homogenizer for 30 min to ensure uniform dispersion. Healthy and uniform greengram seeds were selected and surface-sterilized by immersion in 0.1% sodium hypochlorite solution for 2–3 min, followed by five thorough washes with sterile distilled water to remove the sterilant residue. Sterilized seeds were soaked in the above mentioned concentration of nanoparticle suspensions at a ratio of 1:1 (seed: nano solution) for 3 h at room temperature [26]. Seeds that were primed in sterile distilled water under identical conditions were treated as hydroprimed seeds. Untreated seeds served as control. After priming, the seeds were briefly rinsed with distilled water and air-dried to their original moisture content under shade.

Effect of ZnO nanopriming on seedling development and vigour in greengram

Seed physiological parameters were evaluated using the paper towel roll method with three replicates in accordance with the ISTA 2022 guidelines [27]. The towels were placed in plastic buckets filled with distilled water and incubated in a germination chamber maintained at 25 ± 1°C and 95 ± 2% relative humidity.

Germination percentage (%)

As per the International Seed Testing Association ISTA rules, on the final count day (8^{th} day) the germination count of normally developed seedlings were taken and germination percentage was estimated.

$$\text{Germination percentage} = \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds sown}} \text{x } 100$$

Root Length and Shoot Length (cm)

On the final count day, ten normal seedlings were selected at random from each replication for morphological evaluations. Root length was measured from the collar region to the apex of the primary root using a graduated scale, and the mean length was expressed in centimeters (cm). Subsequently, the same ten seedlings were used to measure shoot length, taken from the collar region to the tip of the primary leaf, with the resulting average recorded in centimeters (cm)

Dry matter Production (g/10 seedlings)

The ten representative seedlings used for root and shoot length measurements were allowed to shade dry for 24 h followed by hot air oven drying at 80^{O}C for 12 hours until a constant mass was reached. The oven-dried seedlings were then weighed using a precision balance, and the mean dry weight was expressed as grams per 10 seedlings

Vigour Index

The seedling vigour indices were determined following a previously established method by Abdul-Baki and Anderson (1973) [28]

Vigour Index I =Germination % x Total seedling length (cm)

Vigour index II = Germination % x Dry matter production (g /10 seedlings)

Influence of ZnO nanopriming on biochemical parameters in greengram

α- Amylase activity (mg maltose min-1)

α- Amylase activity was estimated following Paul et al. (1970)[29]. Pre-germinated seeds (0.5 g) from each treatment were homogenized in 1.8 mL of ice-cold 0.02 M sodium phosphate buffer (pH 6.0) and centrifuged at 20,000 rpm for 20 min at 4 °C. The supernatant was used as the crude enzyme extract. For the assay, 0.2 mL of the extract was mixed with 1.0 mL of 0.067% soluble starch and incubated at 25 °C for 10 min. The reaction was terminated by adding 2.0 mL of iodine–hydrochloric acid reagent (60 mg KI and 6 mg iodine in 100 mL of 0.05 N HCl). Absorbance was measured at 620 nm using a UV–Vis double-beam spectrophotometer (Model 2205), and α-amylase activity was calculated using the standard formula.

$$\alpha - \text{ Amylase activity } = \frac{\text{OD value}}{\text{Volume of sample pipetted out}} \text{x} \frac{1000}{500}$$

Dehydrogenase activity (OD value)

Dehydrogenase activity was determined following the method of Kittock and Law (1968) [30]. Seeds were soaked in distilled water for 7 h and cut longitudinally through the embryo. The embryo halves were stained with 0.5% tetrazolium (TTC) solution prepared in Sorensen's buffer at 40 °C for 2 h in the dark. After staining, the seeds were rinsed with distilled water and immersed in 2-methoxyethanol (methyl cellosolve) overnight in the dark to extract the red formazan pigment. The absorbance of the extract was measured at 470 nm using a UV–Vis spectrophotometer, and the optical density (OD) was recorded as an indicator of dehydrogenase activity.

Catalase activity (μg H_2O_2 min^{-1} g^{-1})

Catalase activity was assayed spectrophotometrically according to Aebi (1984) [31]. Frozen seed tissue (20 mg) was ground in liquid nitrogen and homogenized in 2.0 mL of ice-cold extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA). The homogenate was centrifuged at 15,000 rpm for 20 min at 4 °C, and the supernatant was used as the crude enzyme extract. The reaction mixture (3.0 mL) contained 1.5 mL of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mL of 12.5 mM H_2O_2, 50 µL of enzyme extract and 0.95 mL of distilled water. The decrease in absorbance at 240 nm was monitored at 25 °C for 1 min using an Optima UV–Vis spectrophotometer. Catalase activity was calculated using the H_2O_2 extinction coefficient of 0.0394 mM^{-1} cm^{-1} and expressed as µg H_2O_2 min^{-1} g^{-1}

Peroxidase activity (U mg^{-1} min^{-1})

Peroxidase activity was estimated according to Malik and Singh (1980) [32]. Pre-germinated seeds (500 mg) were homogenized in 5.0 mL of 0.25 M Tris buffer (pH 6.0) and centrifuged at 10,000 rpm for 10 min at 5 °C. The reaction mixture comprised 0.8 mL of the supernatant, 1.0 mL of 0.5% pyrogallol and 1.0 mL of 1% H_2O_2. After incubation at 25 °C for 10 min, the reaction was stopped by adding 1.0 mL of 5% H_2SO_4. The optical density was measured at 420 nm using a UV–Vis spectrophotometer, and peroxidase activity was expressed as U mg^{-1} min^{-1}.

$$\text{Peroxidase activity } = \frac{\text{Difference in OD value}}{10} \text{ x } \frac{1000}{500} \text{ x } 100$$

Statistical Analysis

All experiments were conducted in a completely randomized design (CRD) with three replications. The data were subjected to analysis of variance (ANOVA) using R software. The significance of differences among treatment means was determined by Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. The results are presented as mean ± standard error (SE).

Table 1: Effect of biosynthesized ZnO nanoparticles seed priming on germination, seedling growth and vigour parameters of greengram CO 9

Seed priming Treatments	Germination (%)	Root length (cm)	Shoot length (cm)	Dry matter production (g/10 seedlings)	Seedling vigour index I	Seedling vigour index II
T_0- Control	85.0 ± 0.58^{e}	15.90 ± 0.15^{d}	17.60 ± 0.09^{f}	0.219 ± 0.005	2847 ± 20.08^{d}	19 ± 0.38^{c}
T_1- Hydropriming	88.0 ± 0.58^{cd}	16.02 ± 0.01^{cd}	18.92 ± 0.13^{d}	0.227 ± 0.006	3075 ± 25.03^{c}	20 ± 0.62^{c}
T_2- ZnO NPs @ 100 ppm	94.0 ± 0.58^{b}	16.46 ± 0.11^{bc}	19.98 ± 0.11^{ab}	0.242 ± 0.009	3425 ± 4.63^{b}	23 ± 0.97^{ab}

T_3- ZnO NPs @ 200 ppm	96.0 ± 0.58^{a}	17.12 ± 0.19^{a}	20.43 ± 0.07^{a}	0.251 ± 0.002	3605 ± 14.22^{a}	24 ± 0.14^{a}
T_4- ZnO NPs @ 300 ppm	85.0 ± 0.58^{e}	16.69 ± 0.14^{ab}	19.50 ± 0.18^{bc}	0.244 ± 0.011	3076 ± 31.33^{c}	21 ± 1.03^{bc}
T_5- ZnO NPs @ 400 ppm	90.0 ± 1.15^{c}	16.90 ± 0.15^{ab}	18.30 ± 0.33^{e}	0.231 ± 0.006	3168 ± 57.59^{c}	21 ± 0.53^{bc}
T_6- ZnO NPs @ 500 ppm	86.0 ± 1.00^{de}	14.75 ± 0.29^{e}	19.21 ± 0.11^{cd}	0.227 ± 0.010	2921 ± 40.71^{d}	20 ± 0.61^{c}

Values are mean ± Standard Error. Means followed by a common superscript letter within a column are not significantly different at $p \leq 0.05$ according to Duncan's Multiple Range Test (DMRT). Germination percentages were arcsine-square-root transformed before analysis; original values are shown. Dry matter production: treatment effect not significant (ANOVA, $p = 0.086(>0.05)$); DMRT letters not shown, as all treatments are statistically at par (NS).

Table 2. Effect of biosynthesized ZnO nanoparticles seed priming on biochemical parameters in greengram CO 9

Values represent the mean ± standard error. Data in the same column with different superscripts are significantly

Seed priming treatments	**α-Amylase (mg maltose min-1)**	**Dehydrogenase (OD value)**	**Catalase (μg H_2O_2 min^{-1} g^{-1})**	**Peroxidase (U $mg^{-1}min^{-1}$)**
T_0- Control	0.81 ± 0.01^{e}	0.84 ± 0.01^{f}	1.17 ± 0.02^{f}	0.59 ± 0.01^{e}
T_1- Hydropriming	1.27 ± 0.02^{d}	1.02 ± 0.02^{e}	1.26 ± 0.02^{ef}	0.65 ± 0.01^{d}
T_2- ZnO NPs @ 100 ppm	1.77 ± 0.04^{ab}	1.60 ± 0.03^{b}	1.85 ± 0.04^{c}	0.72 ± 0.01^{c}
T_3- ZnO NPs @ 200 ppm	1.85 ± 0.04^{a}	1.90 ± 0.04^{a}	2.13 ± 0.04^{a}	0.98 ± 0.02^{a}
T_4- ZnO NPs @ 300 ppm	1.65 ± 0.04^{c}	1.41 ± 0.04^{c}	1.99 ± 0.05^{b}	0.81 ± 0.02^{b}
T_5- ZnO NPs @ 400 ppm	1.71 ± 0.03^{bc}	1.38 ± 0.02^{c}	1.52 ± 0.03^{d}	0.70 ± 0.01^{c}
T_6- ZnO NPs @ 500 ppm	1.72 ± 0.03^{bc}	1.22 ± 0.02^{d}	1.34 ± 0.02^{e}	0.68 ± 0.01^{cd}

different at $p < 0.05$ (Duncan's Multiple Range Test)

RESULTS AND DISCUSSION

Mechanism of ZnO NPs formation in biosynthesis method by cell-free supernatant of Methylobacterium radiotolerans

The biosynthesis of ZnO nanoparticles using the cell-free supernatant of Methylobacterium radiotolerans may involve the interaction of Zn^{2+} ions with extracellular biomolecules, including proteins, polysaccharides and other metabolites. Functional groups such as hydroxyl (–OH), carboxyl (–COOH) and amino ($–NH_2$) groups may facilitate zinc ion complexation and contribute to the nucleation and stabilization of the developing particles [20] [33].The formation of zinc-containing precipitates may occur through hydrolysis, followed by thermal conversion into ZnO during calcination at 400 °C. The proposed reactions are:

$$Zn^{2+} + 2OH^{-} \longrightarrow Zn(OH)_2 \xrightarrow{\Delta} ZnO + H_2O$$

The extracellular biomolecules may also act as capping agents, influencing particle growth and preventing excessive aggregation [34]. Thus, the cell-free supernatant may contribute to the initial formation and stabilization of zinc-containing particles, while calcination facilitates the formation of crystalline ZnO nanoparticles.

Characterization of ZnO NPs

The FTIR spectrum of the biosynthesized ZnO nanoparticles using the cell-free supernatant of Methylobacterium radiotolerans (Fig A) showed characteristic absorption bands at 3338.18, 2108.78, 1515.78, 1114.65 and 614.217 cm^{-1}. The broad band at 3338.18 cm^{-1} may be attributed to O–H stretching vibrations, indicating the presence of hydroxyl-containing biomolecules. The absorption band at 1515.78 cm^{-1} may be associated with amide II vibrations, suggesting the possible presence of proteinaceous compounds, while the band at 1114.65 cm^{-1} may correspond to C–O stretching vibrations. Similar functional group assignments were reported by [35] in ZnO nanoparticles synthesised using the cell-free supernatant of Lactobacillus plantarum. The absorption band at 614.217 cm^{-1} is attributed to Zn–O stretching vibrations, supporting ZnO formation, in agreement with [36], who

reported a characteristic band around 615 cm^{-1}. These findings suggest the possible involvement of bacterial biomolecules in nanoparticle synthesis and surface stabilisation.

The X-ray diffraction pattern of the biosynthesized ZnO nanoparticles is shown in Fig. B. The presence of sharp diffraction peaks at 2θ = 31.81° (100), 34.52° (002) and 36.38° (101) confirms the crystalline nature of the ZnO nanoparticles, corresponding to the hexagonal wurtzite structure. The observed diffraction pattern is in close agreement with the findings of [37], who reported characteristic peaks corresponding to the hexagonal wurtzite structure of green-synthesised ZnO nanoparticles. The average crystallite size of the synthesised ZnO nanoparticles was approximately 30.4 nm, as calculated using the Debye–Scherrer equation.

The SEM image of the biosynthesized ZnO nanoparticles is shown in Fig. C. The micrograph revealed irregularly shaped and aggregated nanoparticles, with measured particle dimensions ranging from 39.45 to 87.08 nm, with an average particle size of 61.23 nm.

The EDX spectrum of the biosynthesized ZnO nanoparticles is shown in Fig. D. The presence of prominent zinc and oxygen peaks confirmed the existence of ZnO nanoparticles. The EDX analysis revealed zinc and oxygen atomic percentages of 59.95% and 37.35%, respectively, while their corresponding weight percentages were 83.70% and 12.76%. Minor signals corresponding to molybdenum and chlorine were also detected.

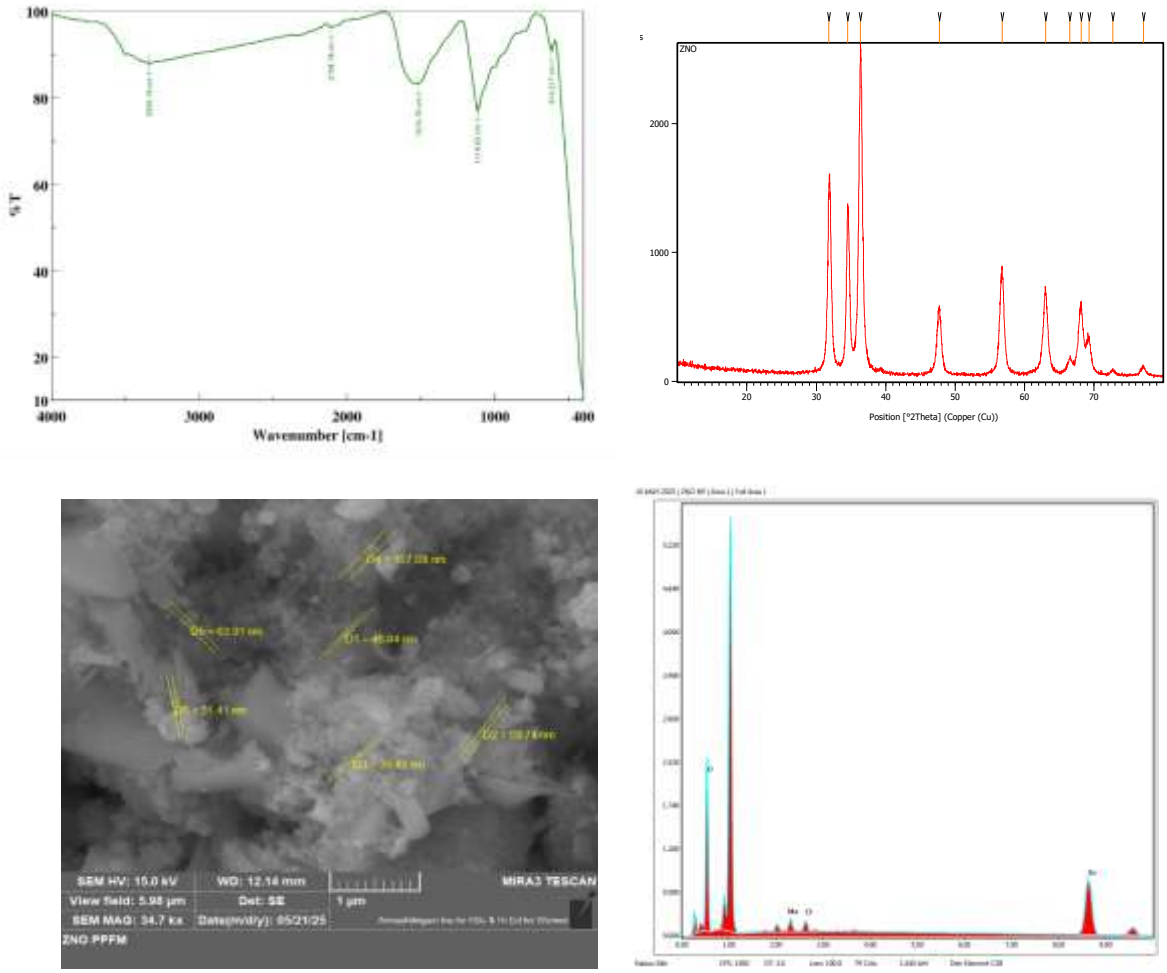


Fig. 3. Characterization of biosynthesized ZnO nanoparticles: (A) FTIR spectrum, (B) XRD pattern, (C) FESEM micrograph and (D) EDX spectrum.

Effect of biosynthesized ZnO nanopriming on physiological traits of greengram CO 9

Seed priming with biosynthesized ZnO nanoparticles significantly influenced germination and seedling growth of greengram CO 9 (Table 1). Among the treatments, ZnO NPs @ 200 ppm (T_3) recorded the highest germination (96.0%), followed by 100 ppm (94.0%), whereas the control recorded 85.0%. The improvement in germination may be attributed to enhanced metabolic activation, reserve mobilisation and enzyme activity during seed imbibition [38] [22] Similar improvements in germination of greengram following ZnO NP priming were reported by [39]

Root and shoot length were also maximum in T_3 (17.12 and 20.43 cm, respectively), representing increases of 7.7 and 16.1 per cent over the control. Zinc plays an important role in enzyme activation, protein synthesis and plant growth regulation, thereby supporting seedling development [40]. Similar improvements in root and shoot growth following ZnO NP application were reported in greengram by [22] [21]. Dry matter production was numerically highest in T_3 (0.251 g/10 seedlings), followed by 300 ppm (0.244 g/10 seedlings); however, the treatment effect

was non-significant. The response at higher concentrations varied, with 500 ppm significantly reducing root length compared with the control, indicating a possible concentration-dependent effect of ZnO NPs.

Seedling vigour index I was significantly highest in T_3 (3605), followed by T_2 (3425) (Fig 4.) while vigour index II was highest in T_3 (24), which was statistically at par with T_2 (23). The improvement in vigour indices could be attributed to the combined enhancement of germination and seedling growth. Comparable improvements in seedling vigour following ZnO NP priming have been reported in greengram [22], blackgram[41] and rice [42]. The reduced response at higher concentrations may be associated with excessive zinc availability and possible oxidative stress, although these mechanisms were not directly examined in the present study. Overall, biosynthesized ZnO NPs @ 200 ppm was the most effective treatment for improving germination, seedling growth and vigour of greengram CO 9.

Effect of biosynthesized ZnO nanopriming on biochemical traits of greengram CO 9

Seed priming with biosynthesized ZnO nanoparticles significantly influenced the biochemical parameters of greengram CO 9 (Table 2). Among the treatments, ZnO NPs at 200 ppm (T_3) recorded the highest activities of α-amylase (1.85), dehydrogenase (1.90), catalase (2.13) and peroxidase (0.98), representing increases of 128, 126, 82 and 66 per cent, respectively, over the control. T_3 was statistically superior to all other treatments for dehydrogenase, catalase and peroxidase activities. However, its α-amylase activity was statistically on par with that of T_2 (100 ppm). Hydropriming also improved α-amylase, dehydrogenase and peroxidase activities over the control, whereas catalase activity under hydropriming was statistically on par with the control.

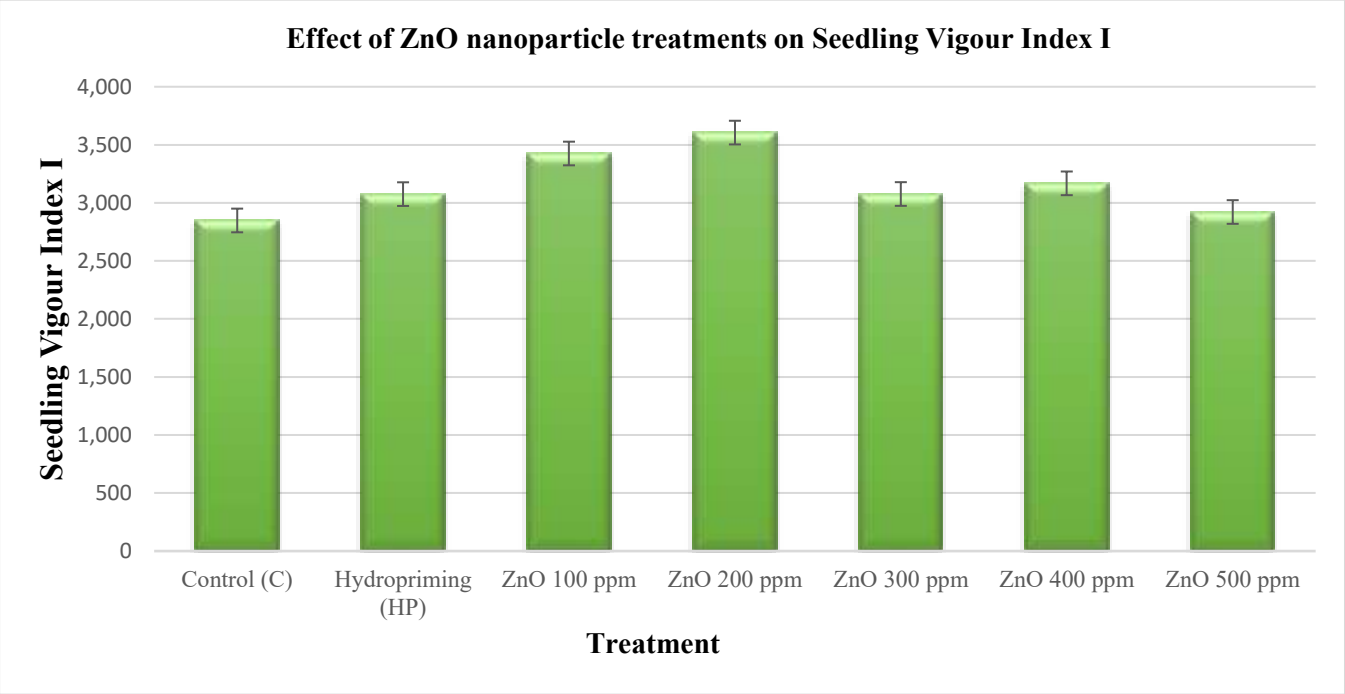


Fig. 4. Effect of different concentrations of biosynthesized ZnO nanoparticles on Seedling Vigour Index I of greengram (Vigna radiata L.) cv. CO 9.

The increased α-amylase activity in primed seeds indicates enhanced starch hydrolysis, which supplies soluble sugars required for energy production during germination. Dehydrogenase activity reflects respiratory metabolic activity and is commonly associated with seed viability and vigour. Catalase and peroxidase are important antioxidant enzymes that scavenge hydrogen peroxide and help maintain cellular redox balance. The improved activities of these enzymes at 200 ppm may therefore have contributed to better metabolic activation and seedling performance. Zinc plays important roles in several enzymatic processes and contributes to cellular redox regulation and antioxidant defence [43] [44]. Similar improvements in germination-associated enzyme activities following nanoparticle-based seed priming have been reported by [45]

A decline in some enzyme activities was observed at ZnO NP concentrations above 200 ppm, indicating that the response was concentration-dependent. At 500 ppm, dehydrogenase and catalase activities decreased to 1.22 and 1.34, respectively, compared with 1.90 and 2.13 at 200 ppm. However, α-amylase activity remained comparatively high across the 300–500 ppm treatments. The reduced response at higher concentrations may be associated with an imbalance in zinc uptake or nanoparticle-related effects on cellular metabolism. Excess zinc may also interfere with growth-regulating processes, including tryptophan and auxin biosynthesis [46]. Nevertheless, these mechanisms were not directly examined in the present study. Overall, ZnO NP seed priming at 200 ppm was the most effective treatment for enhancing the measured biochemical activities of greengram CO 9.

CONCLUSION

ZnO nanoparticles were successfully biosynthesized using the cell-free supernatant of Methylobacterium radiotolerans and characterized through FTIR, XRD, SEM and EDX analyses. The characterization results supported the formation of crystalline ZnO nanoparticles with a hexagonal wurtzite structure, irregular morphology and the presence of zinc and oxygen. Furthermore, the effect of seed priming with biosynthesized ZnO nanoparticles on germination, seedling growth, vigour and biochemical parameters of greengram CO 9 was evaluated. Among the different concentrations tested, seed priming with ZnO nanoparticles at 200 ppm exhibited the best overall performance, enhancing germination, seedling growth, vigour indices and biochemical enzyme activities. Thus, the findings highlight the potential of Methylobacterium radiotolerans-mediated ZnO nanoparticles as a promising biological approach for seed priming and improving seedling performance in greengram.

The promising germination, seedling growth and biochemical responses observed in greengram CO 9, particularly with seed priming using 200 ppm biosynthesized ZnO nanoparticles, provide a basis for further investigations into their agricultural applications. Although the present study demonstrated the beneficial effects of Methylobacterium radiotolerans-mediated ZnO nanoparticles under the experimental conditions, future studies could explore their effectiveness in other pulse and cereal crops to establish their suitability across different crop species. Further investigations involving different nanoparticle concentrations, seed priming durations and storage conditions may help optimise the application protocols for improved seed performance.

Moreover, future research could focus on evaluating the influence of biosynthesized ZnO nanoparticles under various abiotic stress conditions, including drought, salinity and high-temperature stress, to understand their potential role in improving crop establishment and stress tolerance. Detailed investigations into zinc uptake, translocation, nutrient assimilation, antioxidant metabolism and molecular responses would provide better insights into the physiological mechanisms underlying nanoparticle-mediated seed enhancement. In addition, field-level evaluations, nanoparticle persistence and environmental safety assessments are essential to establish their practical applicability. Such studies could contribute to the development of eco-friendly, microbe-mediated nanopriming approaches for improving seed quality, crop establishment and sustainable agricultural production.

CRediT authorship contribution statement

Tharani N; Writing original draft. Manonmani V; Conceptualization and Validation. Pon. Sathya Moorthy; Methodology and Supervision. Raja K, Senthil Kumar M, and Johnson I: Reviewing and Visualization. All authors have read and approved the research article.

Funding

The authors gratefully acknowledge Incotec India Pvt. Ltd. for providing financial support to carry out this research work.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors sincerely thank the Department of Seed Science and Technology, Department of Microbiology and Centre for Agricultural Nanotechnology, Tamil Nadu Agricultural University, Coimbatore, India, for providing the necessary facilities and support to carry out this research work. The authors also gratefully acknowledge Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, India, for providing the instrumentation facilities to perform the characterization analyses.

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