

## Research Article

# Mechanistic Insight of the Antifungal Potential of Green Synthesized Zinc Oxide Nanoparticles against *Alternaria brassicae*

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Received 20 February 2022; Accepted 24 March 2022; Published 2 May 2022

Academic Editor: Palanivel Velmurugan

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*Alternaria brassicae* is a necrotrophic fungus causes *Alternaria* blight disease in oilseed mustard crop. There is a 47% loss of the mustard crop due to the *Alternaria* blight disease. To control this disease, various chemical fungicides have been used till date which are harmful to our environment. Zinc oxide (ZnO) nanoparticles synthesized from *Terminalia bellerica* have been reported to have significant antifungal potential against *A. brassicae* at 200 ppm concentration. In the present study, the effect of green synthesized zinc oxide (Tb-ZnO) nanoparticles, chemically synthesized ZnO nanoparticles, and chemical fungicides on *A. brassicae* has been anticipated by analysing changes in cytomorphology characteristics, biochemical constituent, and stress enzymes of *A. brassicae*. Cytomorphological studies by transmission electron microscopy and scanning electron microscopy have shown the complete disintegration of cell wall, cell membrane, and cytoplasmic content at 200 ppm concentration of Tb-ZnO nanoparticles. Decrement in biochemical constituents and changes in activity of stress enzymes in Tb-ZnO nanoparticles treated cell confirm the toxicity of nanoparticles at 200 ppm concentrations. Hence, on the basis of all these results, the mechanism of action of Tb-ZnO nanofungicides on *A. brassicae* has been hypothesized in the present study. This study confirms how nanoparticles inhibit the growth of *A. brassicae* and suggested the use of nanofungicides.

## 1. Introduction

Fungi is a very large kingdom acknowledged for its global presence in any type of habitation. Fungal diseases in the plants have become thoughtful alarm as 70% of loss in economically important crops productivity is due to these diseases. Among various economically important crops, oilseed mustard is the second most important oilseed crop after groundnut. Fungal diseases of oilseed mustard are white rust, powdery mildew, downy mildew, *Sclerotinia* stem rot, and *Alternaria* leaf spot or *Alternaria* blight or blight disease. Among these fungal diseases, blight disease caused by necrotrophic fungi, *Alternaria brassicae* has been reported worldwide and is a noxious disease of mustard oilseed causing 47% losses in yield without transferable resistance [1]. Chemical fungicides are being used for a long

time but this becomes a subject of public health concern now because chemical fungicides are harmful to our environment, for the nontarget organism, causes the development of resistant pathogens and also they are carcinogenic in nature [2]. Moreover, they have shown resistance even at higher concentrations of these formulations and sometimes require multiple applications. Therefore, these resistant pathogens have become the biggest threat for the farmers [3]. Hence, development of new alternative to eradicate the harmful impact of these chemicals on human health and for food safety and to achieve sustainable agriculture is needed [4].

Biocontrol agents are the results of one of such efforts that may provide an environment-friendly alternative to poisonous fungicides which are not harmful to the environment [5]. Disadvantages of using microorganisms as

biological control include maintenance of hygienic conditions, lack of consistency, take several years to check host specificity, cannot remove the pest completely as they destroy their food source, and being expensive during the field survey and early testing stages [6]. Also, the development of resistance to biocontrol agent or chemical fungicides in pathogenic fungi is a thoughtful alarm [2].

A significant alternative to these formulations could be metal or nonmetal oxide nanoparticles. Nanotechnology is growing rapidly as nanoparticles have a vast area of applications owing to their type, size, shape, and route of synthesis. In the last decade, different metal nanoparticles have been synthesized through physical, chemical, and biological methods. These nanoparticles have applications in areas like physics, chemistry, pharmaceutical science, material science, medicine, and agriculture [7, 8]. The significant results in other fields opened up a lot of scope in the agriculture field also [9]. Scientists have proposed the use of nanoparticles for the eradication of phytopathogens after getting the significant proof of potential biostatic and biocidal properties of various types of nanoparticles against human and animal pathogens [10, 11].

Among all the nanoparticles, zinc oxide nanoparticles (ZnO NPs) are one of the most widely used nanoparticles due to their optical, photocatalytic, and antimicrobial properties [12]. ZnO NPs have been generally recognized as safe by the U.S. Food and Drug Administration (FDA, 2016) [7] and reported as antibacterial, antiviral, and antifungal agents [13, 14]. The antimicrobial property of biologically synthesized ZnO NPs has been proven more effective in comparison to their chemical counterparts [15, 16]. The mechanistic approach of ZnO NPs to inhibit the growth of microorganisms is induction of oxidative stress which results in the production of reactive oxygen species, disruption of the cell membrane due to internalization, accumulation of nanoparticles inside the cell, and damage of nucleic acid [17].

In our previous study, the phytofabricated zinc oxide nanoparticles (Tb-ZnO NPs) as nanofungicides have been reported to have antifungal activity against *A. brassicae*. Synthesized Tb-ZnO NPs have shown significant antifungal potential at 200 ppm as compared to commercially available chemical fungicides and chemically synthesized ZnO NPs. The changes in fungal hyphae morphology and reduction in spore germination were reported by microscopic studies [18]. To get more insight into the mechanism of action of nanoparticles against the fungal cell, the present work has been done to study the mechanistic approach of Tb-ZnO NPs against *A. brassicae* by analysing the cytomorphological, biochemical constituent and stress enzyme.

## 2. Material and Methods

**2.1. Chemicals.** Chemically synthesized zinc oxide nanoparticles (C-ZnO; size  $\approx 30$  nm) were purchased from SRL Pvt Ltd. Commercially available fungicides mancozeb and propiconazole were purchased from online sources. Potato dextrose broth and all other chemicals used in the study are of analytical grade.

### Treatments Used in the Study

- T1- Control (Negative control)
  - T2- Chemical fungicide 1 - Mancozeb
  - T3- Chemical fungicide 2 - Propiconazole
- } (Positive control)
- T4- Chemically synthesized ZnO nanoparticles (C-ZnO) at the concentration of 50, 100, 150, 200 ppm
  - T5- Phytofabricated ZnO nanoparticles (Tb-ZnO) at the concentration of 50, 100, 150, 200 ppm

**2.2. Phytofabrication, Characterization, and Antifungal Potential of Zinc Oxide Nanoparticles.** ZnO nanoparticles were synthesized from aqueous plant leaf extract of *Terminalia bellerica*. Characterization of synthesized ZnO NPs were done by UV-vis spectrophotometer, dynamic light scattering, zeta potential, Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The antifungal property of ZnO nanoparticles synthesized from aqueous plant leaf extract of *Terminalia bellerica* (Tb-ZnO) was determined by poisoned food technique and microscopic studies of hyphae and spores. All experimental details related to synthesis process have been given in previous study [18].

**2.3. Biochemical Constituents.** For the analysis of effect of different treatments (T1-T5) on biochemical constituent, the fungal mat of *Alternaria brassicae* was grown in different treatments (T1-T5) for 10 d. And further fungal mat or filtrate has been used as per requirement of the study.

**2.4. Protein.** Estimation of protein content in the *A. brassicae* was done by the Lowry method. It is the most commonly used method to estimate the protein in the biological samples. The Lowry method comprises of alkaline copper sulphate reagent (48 mL of 2% sodium carbonate in 0.1 N sodium hydroxide +1 mL of 1% sodium potassium tartrate +1 mL of 0.5% copper sulphate) with the Folin-Ciocalteu phenol (FPR) reagent (FPR: distilled water; 1:1), which interacts with the tryptophan residue present in the protein. For this, 10 mg of fungal culture was taken in mortar pestle and grinded with 20 mL of phosphate buffer of pH 7.4 (0.8 g of NaCl, 0.02 g of KCl, 0.144 g of  $\text{NaH}_2\text{PO}_4$  and 0.0245 g of  $\text{KH}_2\text{PO}_4$  + 100 mL of double distilled water). Above mixtures were centrifuged at 10,000 rpm for 10 min. After centrifugation, 0.2 mL of supernatant was taken from tube and final volume was made 1 mL by adding distilled water. Then, 4.5 mL of alkaline copper sulphate reagent was added and incubated at room temperature for 10 min. After 10 min of incubation at room temperature, 0.5 mL of FPR was added in tube and incubated again at room temperature for 15-20 min. After visible blue colour development, the absorbance was taken at 660 nm [19]. This procedure was followed for all treatments (T1-T5) given to the *A. brassicae*. Standard curve was prepared by Bovine Serum Albumin (BSA) [20].

**2.5. Extracellular and Intracellular Polysaccharides.** For the estimation of extracellular polysaccharides (EPS), a 10-d-old fungal mat was removed from the culture broth through filtration with the help of Whatman no. 1 filter paper. For the precipitation of EPS present in the filtrate, 1 mL of 10%

trichloro acetic acid (TCA) was added to the above filtrate. After this, 1 mL from each treatment (T1-T5) was used for EPS analysis by the anthrone method with the help of UV-vis spectrophotometer at 620 nm. This procedure was repeated with all the treatments (T1-T5). For the estimation of intracellular polysaccharides (IPS) of fungus (*Alternaria brassicae*) treated with T1-T5, the 5 mg of fungal biomass from each treatment was taken and crushed with 10 mL of phosphate buffer (pH 6.5). All the suspensions were centrifuged at 7000 rpm for 20 min. In the supernatants of each treatment, 1 mL of 10% TCA was added to see the precipitation of proteins. After this, 1 mL of supernatant from each treatment (T1-T5) (free from protein) was used for intracellular polysaccharide analysis by the anthrone method with the help of UV-vis spectrophotometer at 620 nm [21]. The EPS and IPS contents were determined by using standard curve of glucose as  $\text{mg mL}^{-1}$ .

**2.6. Chitin.** For the chitin estimation, procured fungal mat was dried in hot air oven at 40°C for 8-10 h. The chitin estimation was done by two phase extraction studies. After drying, 0.5 g of fungal mat was dissolved in 1M of 15 mL NaOH and kept in water bath at 40°C for 2 h. After 2 h of incubation in water bath, NaOH was discarded with the help of pipette and fungal mat was washed with water. After washing, 1 mL of potassium permanganate (10 g/L) was added and incubated at room temperature for 1 h. Then, oxalic acid (10 g/L) was added for the decolourization of the fungal mat. After decolourization, oxalic acid was discarded and dried mass of chitin was weighed [22]. This procedure was followed for all the treatments (T1-T5).

## 2.7. Enzyme's Analysis

**2.7.1. Cellulase Enzyme.** For the quantitative analysis of cellulase enzyme activity in treated fungi (*Alternaria brassicae*), the modified Mendel's medium was used containing 0.1% peptone, 0.03% urea, 0.0016%  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0014%  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.14%  $(\text{NH}_4)_2\text{SO}_4$ , 0.03%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01%  $\text{CaCl}_2$ , 0.0029%  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.2%  $\text{KH}_2\text{PO}_4$ , 1% cellulose and pH of medium was maintained at 5.5. Then, 10 mL from above media was transferred in the Erlenmeyer flasks and different concentrations (T1-T5) were added to check cellulase production. After autoclaving all the media in the flasks, 5 mg of fungal mat grown for 10 d was transferred to each flask and incubated at 28°C at 150 rpm for 10 d. On 10<sup>th</sup> d, flasks were harvested by filtration through Whatman filter paper number 1. The filtrates were centrifuged at 1000 rpm in 4°C for 10 min and supernatants were used for enzyme activity assay as crude enzyme [23].

(1) *Exo-1-4-Glucanase Assay.* In this assay, 1 mL of crude enzyme was incubated with 1 mL of 0.5% cellulose in 0.1 M citrate phosphate buffer having pH 4.8 for 30 min at 50°C. After 30 min, resulted reducing sugar was measured using DNS (Di-Nitro Salicylic acid) method.

(2) *Endo-1-4-Glucanase Activity or CMC Case Assay.* In this assay, 1 mL of crude enzyme was incubated with 1 mL of 1% carboxy methyl cellulase in 0.1 M sodium acetate buffer having pH 5 for 30 min at 50°C. After 30 min, resulted reducing sugar was measured using DNS method [24].

For the DNS preparation, 30 g of potassium sodium tartrate and 1 g of DNS were dissolved in 50 mL of distilled water, and then, 20 mL of 2 N NaOH was added. Then, final volume was made up to 100 mL by adding distilled water [25].

One unit (U/mL) of cellulase activity is defined as the amount of cellulase required to liberate 1 micromole of reducing sugar (D-glucose) per min under the assay conditions. Cellulase activity was calculated using the formula.

$$\text{Enzyme activity} = \frac{\Delta E \times Vf}{Vs \times \Delta t \times \sum \times d}, \quad (1)$$

where  $\Delta E$  is the absorbance at 540 nm,  $Vf$  is the final volume of reaction mixture including DNS,  $Vs$  is the crude supernatant (mL) containing cellulase used,  $\Delta t$  is the incubation time for hydrolysis,  $\sum$  is the extinction coefficient of glucose (0.0026), and  $d$  is the diameter of cuvette.

**2.7.2. Lipase Enzyme.** For the analysis of lipase activity of *Alternaria brassicae* at different concentrations of T1-T5, the production medium containing glucose 0.1%, olive oil 3%,  $\text{NH}_4\text{Cl}$  0.5%, yeast extract 0.36%,  $\text{H}_2\text{HPO}_4$  0.1%,  $\text{MgCl}_2$  0.01%, and  $\text{CaCl}_2$  0.04% was prepared with different concentrations of T1-T5. 10 mg of fresh culture of *A. brassicae* was added in each flask and incubated for 10 d. After 10 d, filtrate was filtered with Whatman filter paper no. 1 and supernatant was used for enzyme assay for analysing extracellular lipase activity. For intracellular lipase activity, mycelial mass was grinded with 10 mL of phosphate buffer having pH 7 and filtered with Whatman filter paper no. 1. Obtained filtrate was used for further analysis of intracellular activity of lipase.

For enzyme assay, 1 mL of the filtrate (extracellular lipase and intracellular lipase) was added in 10 mL of 10% olive oil in 10% gum acacia, 2 mL of 0.6%  $\text{CaCl}_2$ , and 5 mL of 1 M phosphate buffer. Then, above mixture was incubated for 1 h at 150 rpm. After incubation, 20 mL of alcohol: acetone (1:1) was added in the flask to stop reaction. Liberated fatty acid was titrated with 0.1 N NaOH using phenolphthalein as an indicator. The end point should be light pink in colour [26, 27]. The enzyme activity was calculated by the following formula:

$$\text{Enzyme activity} = \frac{(\text{NaOH})(\text{Molarity of NaOH})(1000)(2)(df)}{(1)}, \quad (2)$$

where NaOH is the volume of NaOH (mL) used for the treatment—volume of NaOH (mL) used for control; 1000 is the conversion factor from milliequivalent to microequivalent; 2 is the time conversion factor from 30 min to 1 h;  $df$  is the dilution factor; and 1 is the volume of enzyme (mL) used.

### 2.7.3. Stress Enzymes.

(1) *Superoxide Dismutase (SOD)*. Superoxide dismutase (EC 1.15.1.1) enzyme activity was analysed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) as the method suggested by Beuchamp and Fridovich. For this 3 mL of assay mixture (50 mM phosphate buffer of pH 7.8, 75  $\mu$ M NBT, 13 mM methionine, 2 mM riboflavin, 0.1 mM EDTA) and enzyme extract was incubated for 30 min under a fluorescent lamp. The absorbance was recorded at 560 nm ( $\epsilon = 100 \text{ mM}^{-1} \text{ cm}^{-1}$ ) [28].

(2) *Catalase*. Catalase activity was analysed by the Aebi (1984) method. In this method, 3 mL of reaction mixture containing 50 mM phosphate buffer (pH -7), 20 mM  $\text{H}_2\text{O}_2$  was added in 0.5 mL of enzyme extract and absorbance was measured at 240 nm. The molar extinction coefficient used for the enzymatic activity was  $\epsilon = 0.4 \mu\text{M}^{-1} \text{ cm}^{-1}$  [28].

2.8. *Effect of Tb-ZnO NPs on the Cytomorphology of A. brassicae through SEM and TEM Analyses*. To determine the changes in the surface and internal morphology of treated *A. brassicae*, the SEM and TEM, respectively, were used. From the previous studies, 200 ppm concentration of Tb-ZnO NPs found to be more effective against *A. brassicae*. Therefore, for the further studies, *A. brassicae* treated with C-ZnO nanoparticles (200 ppm) and Tb-ZnO nanoparticles (200 ppm) were used. *A. brassicae* without any treatment (negative control) and treated with mancozeb and propiconazole (positive control) was used throughout the study.

For SEM analysis, sample was taken from the culture grown on potato dextrose broth for 10 d. Treated fungal mats were cut with a sterile surgical knife in the shape of square block (5 mm) and then washed with autoclaved distilled water by centrifugation (7000 rpm for 10 min) for the removal of nutrient broth from the fungal mat. After water washing, fungal mats were fixed with 2% glutaraldehyde for 4 h at 4°C. Following fixation, the fungal mats were dehydrated in graded ethanol series 30, 40, 50, 60, 70, 80, 90, and 100% and kept at room temperature for drying. The dried samples were coated with thin layer of gold for making the samples conductive through sputter coating procedure (Quorum). After coating, samples were studied through scanning electron microscope (ZEISS EVO) [29].

For TEM analysis, treated fungal mats were cut with the sterile surgical knife in the shape of square block (1 mm) and then washed with autoclaved distilled water by centrifugation (7000 rpm for 10 min) and fixed with 2% glutaraldehyde for 4 h at 4°C. After fixation, the samples were stored in 0.1 M phosphate buffer saline of pH 7.4 for 10-12 h and sent for further block preparation and TEM (TECNAI (HR-TEM)) analysis to the Sophisticated Analytical Instrumentation Facility (DST), AIIMS TEM facility, New Delhi [30].

## 3. Results

3.1. *Synthesis, Characterization, and Antifungal Potential of Zinc Oxide Nanoparticles*. Synthesis of ZnO NPs were done by aqueous plant leaf extract of *Terminalia bellerica* (*T. bel-*

*lerica*). UV-vis spectrophotometry, zeta size and potential, Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM), and Transmission Electron Microscopy (TEM) analysis revealed the hexagonal-shaped 22 nm zinc oxide nanoparticles (Tb-ZnO NPs). Antifungal potential of Tb-ZnO NPs was analysed by poisoned food technique. The results suggested that 200 ppm concentration of Tb-ZnO NPs was effective in inhibiting the growth of *A. brassicae* causative agent of blight disease in mustard crop. The study included the comparative analysis of C-ZnO nanoparticles, Tb-ZnO nanoparticles, and commercially available chemical fungicides. Poisoned food techniques results showed that Tb-ZnO NPs were very effective in inhibiting the growth of *A. brassicae* up to 95% at 200 ppm concentration. Microscopic studies also confirmed the absence of spores and deformed fungal hyphae posttreatment. The detailed experiments and results of this study have been already reported in our previous study which is shown in Figure 1 [18].

3.2. *Effect of Tb-ZnO Nanoparticles on Biochemical Constituents of A. brassicae*. Proteins, polysaccharides, and chitin are the main building block of fungal cell wall and provide rigidity to the cell. Polysaccharides/glucans are carbohydrates and most abundant in the fungi used for cell's immediate energy demands. Protein plays critical role in variety of functions of cell both structural and catalytic. Proteins are type of intracellular organic molecules made from amino acid, whereas chitin is an essential component of fungal cell wall. When chitin synthesis is disrupted, the wall becomes disordered and the fungal cells become malformed and osmotically unstable [31]. All these components of cell wall have different functions including maintenance of cell shape, fungal cell protection from external stresses, absorption of molecules, adhesion, signal transmission, and formation of cell wall component [32].

3.3. *Proteins*. The effect of different concentrations of T1-T5 on protein concentration of *A. brassicae* was analysed and is presented in Figure 2. The protein concentration in the negative control (T1) and in positive control (T2-T3) was found to be 3.54, 2.72, and 2.21 mg/mL, respectively. In case of treatments with different concentrations of C-ZnO nanoparticles (50, 100, 150, and 200 ppm) (T4), the concentration of protein was found to be 3.17, 2.53, 2.31, and 2.27 mg/mL, respectively, whereas the treatment with different concentrations of Tb-ZnO nanoparticles (50, 100, 150, and 200 ppm) (T5), the concentration of protein was found to be 3.05, 1.72, 1.43, and 1.29 mg/mL, respectively. From the results obtained, it was observed that minimum concentration of protein was present in the *A. brassicae* treated with Tb-ZnO nanoparticles (200 ppm) as compared to all other treatments. 65% reduction in the protein production was found in the presence of Tb-ZnO nanoparticles. This confirms the inhibitory action of nanoparticles on protein synthesis [33].

3.4. *Polysaccharides*. Figure 3 represents the impact of different concentration of T1-T5 treatments on the intracellular and extracellular polysaccharides of *A. brassicae*. The intracellular



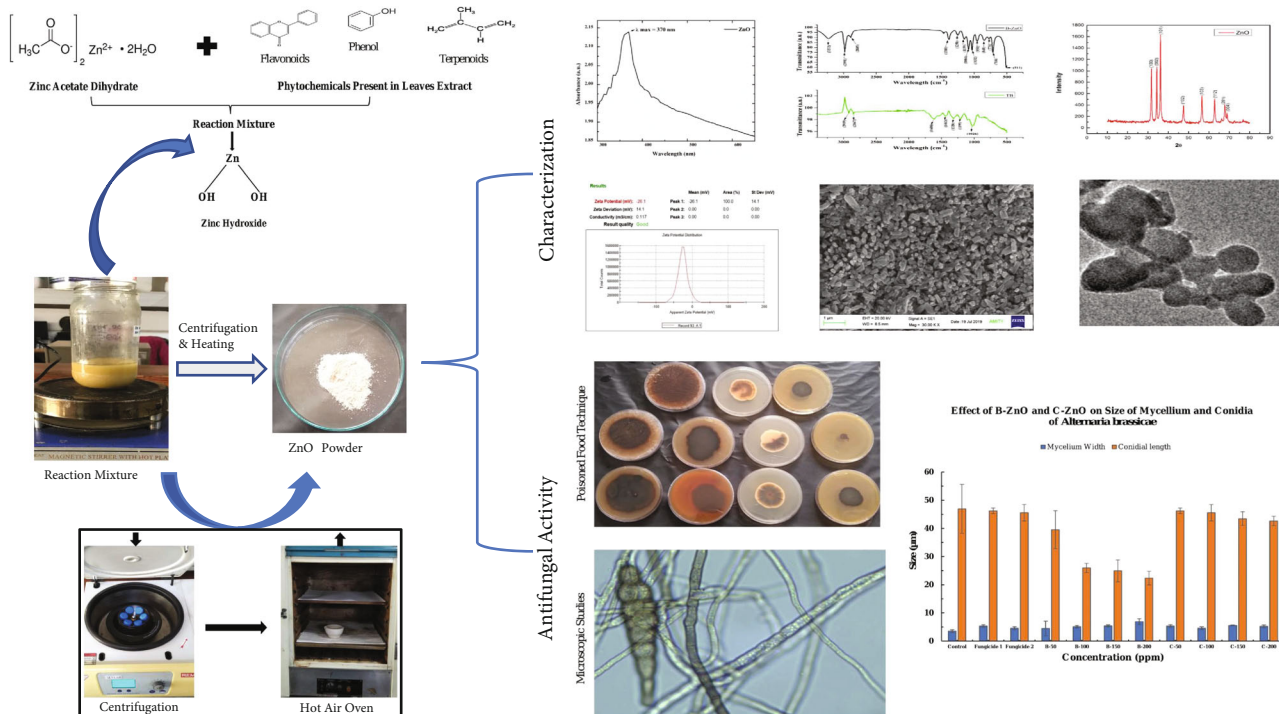


FIGURE 1: Synthesis, characterization, and antifungal potential of Tb-ZnO nanoparticles [18]

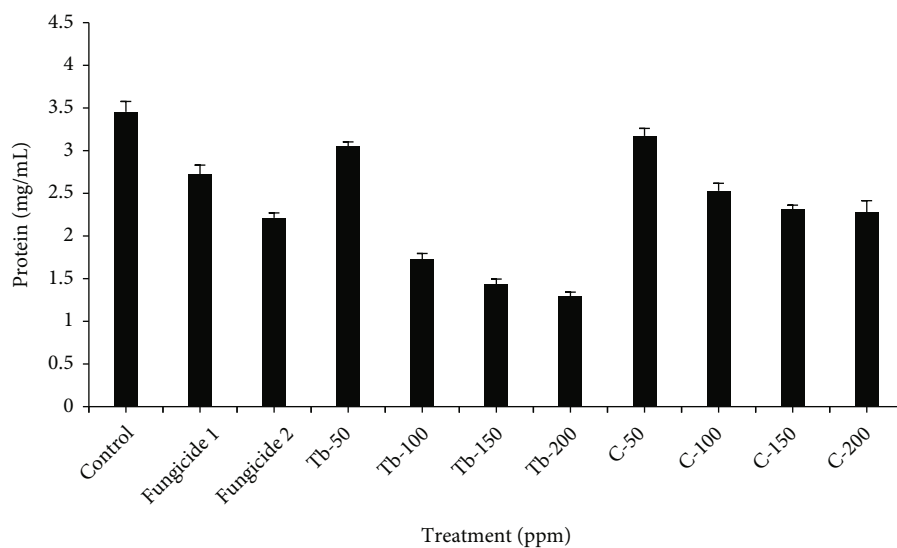


FIGURE 2: Effect of different concentrations of T1-T5 on protein concentration of *Alternaria brassicae*.

polysaccharide concentration in the T1 and T2-T3 was found to be 0.493, 0.310, and 0.346 mg/mL, respectively, and extracellular polysaccharide concentration was found to be 0.785, 0.323, and 0.346 mg/mL, respectively. In case of treatments with different concentrations of T4 (50-200 ppm), the concentration of intracellular polysaccharides was estimated as 0.461, 0.334, 0.157, and 0.159 mg/mL, respectively, and extracellular polysaccharide concentration was analysed as 0.762, 0.798, 0.778, and 0.630 mg/mL, respectively, whereas the treatment with different concentrations of T5 (50-200 ppm), the concentration of intracellular polysaccharides was estimated as 0.337, 0.168, 0.122,

and 0.071 mg/mL, respectively, and extracellular polysaccharide concentration was analysed as 0.781, 0.801, 0.327, and 0.094 mg/mL, respectively. 86% and 88% reduction in intracellular and extracellular polysaccharide content was found after treatment with Tb-ZnO nanoparticles (200 ppm). This also confirms the inhibitory action of nanoparticles against the test fungi by inhibiting the synthesis of polysaccharides [10, 33].

**3.5. Chitin.** Chitin is an important component of fungal cell wall and its concentration increases in response to the stress. It has been reported that chitin content increases to provide

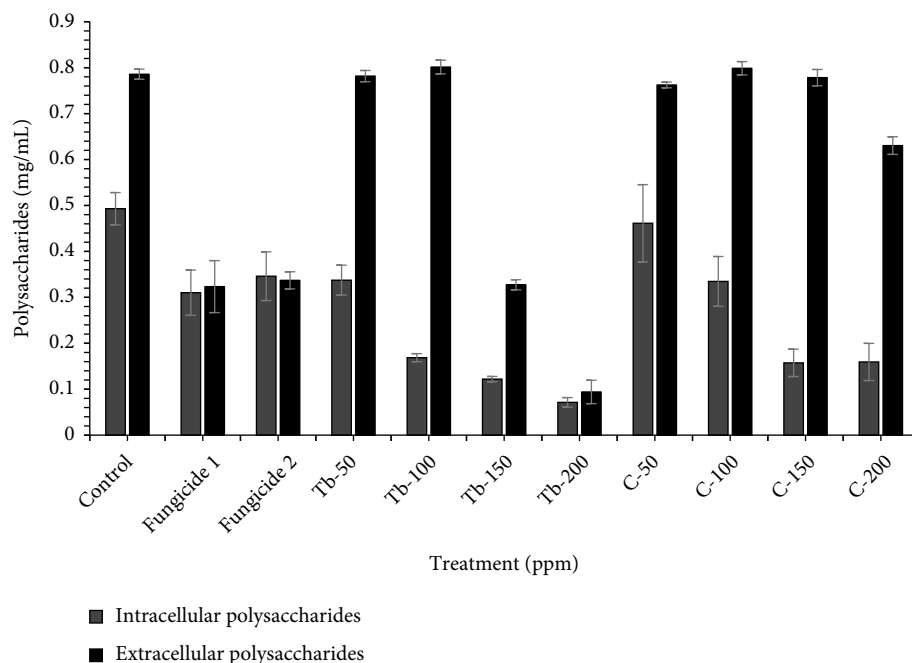


FIGURE 3: Effect of different concentrations of T1-T5 on intracellular and extracellular polysaccharides of *A. brassicae*. Bars are the means of three replicates and vertical lines represent the standard error ( $\pm$ SE).

the cell wall rigidity to combat the external stress [34]. A common response to cell wall damage is strengthening of the wall by the production of excess chitin, primarily by the class IV enzymes such as ScChs3 and CaChs3. Figure 4 represents the chitin content of *A. brassicae* in different concentrations of T1-T5 treatment. The chitin content in T1 and T2-T3 was found to be 0.60, 0.42, and 0.36 mg/mL, respectively. In case of treatments with different concentrations of T4 (50-200 ppm), the content of chitin was estimated as 0.71, 0.61, 0.54, and 0.44 mg/mL, respectively, whereas the treatment with different concentrations of T5 (50-200 ppm), the content of chitin was observed as 0.70, 0.75, 0.55, and 0.35 mg/mL, respectively. From the results obtained, it has been observed that minimum content of chitin was present in the *A. brassicae* treated with Tb-ZnO NPs (200 ppm) as compared to all other treatments. Increase in chitin content in the initial concentration of treatment (T4-T5) shows the defence mechanism of *A. brassicae* against nanoparticles stress. While the increased concentration of nanoparticles might damage the chitin synthesis, machinery leads to decrease in chitin content of test fungi at MIC concentrations that is 200 ppm concentration of nanoparticles used for the treatment.

From Figure 4, it can be concluded that in the starting concentrations of nanoparticles, the *A. brassicae* was in stress as content of chitin was maximum up to 100 ppm concentration of Tb-ZnO NPs. Post this concentration, there was deep fall in the chitin content, this might be due the inactivation of ScChs3 and CaChs3 enzymes [31]. Chitin is very essential component of fungal cell wall and inhibition of chitin has been proposed as an attractive target for anti-fungal therapy.

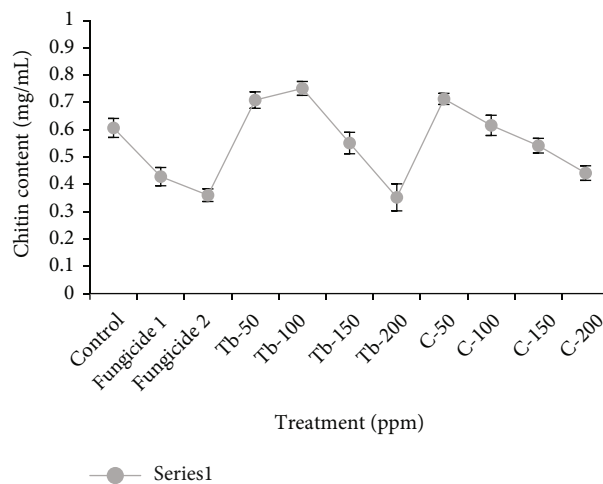


FIGURE 4: Effect of different concentrations of T1-T5 on chitin content of *Alternaria brassicae*. Bars are the means of three replicates and vertical lines represent the standard error ( $\pm$ SE).

**3.6. Effect on Host Cell Wall-Degrading Enzymes.** Plant cells are confined by a rigid extracellular matrix known as cell wall that plays a critical role in biochemical, mechanical, and physiological functions [35]. This rigid extracellular matrix provides tensile strength, mechanical support, modulating cell expansion, permitting elevated internal turgor pressures, and eventually determining cell size and shape [36]. Cell walls have several other important functions such as cell to cell communication, contact with the external environment, and cell adhesion and also provide chemical-physical barrier to the loss of water and to the phytopathogenic attack. Phytopathogens

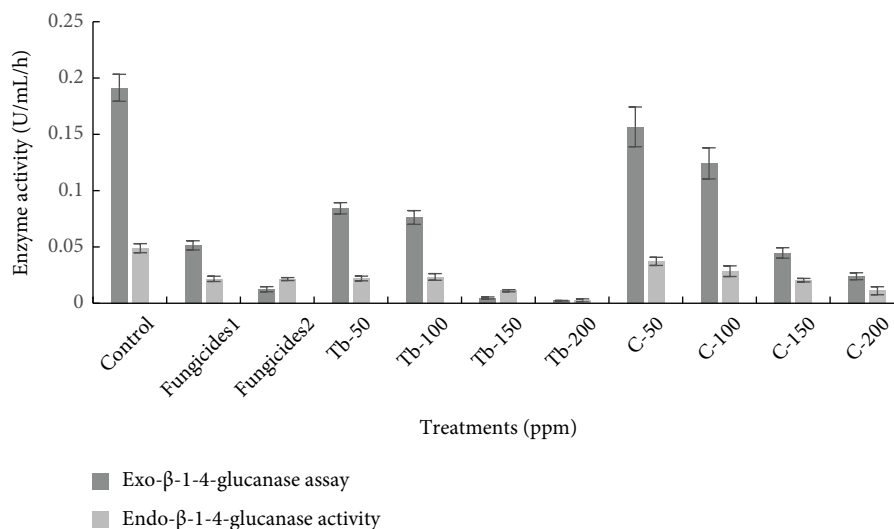


FIGURE 5: Exoglucanase and endoglucanase activity of *Alternaria brassicae* treated with different concentrations of T1-T5. Bars are the means of three replicates and vertical line represents the standard error ( $\pm$ SE).

have different types of cell wall-degrading enzymes to degrade the structural component of cell wall to gain entry in the host cell and to use carbon sources and nutrients for their own survival [37]. As compared to other phytopathogens, fungus has been reported to secrete a variety of cell wall-degrading enzymes that play major role in pathogenicity [38]. The two major types of cell-degrading enzymes are cellulase and lipase. Cellulase and lipase have been reported to play major in virulence of *Alternaria* species [39–41]. Lipases are involved in penetration of plant cell barrier (waxes and cuticles) by the *Alternaria* and then degrade the internal stored lipids [42]. Virulence of *Alternaria* species has been reported to be associated with these cell wall-degrading enzymes [41].

**3.7. Cellulase Activity.** Cellulases are the group of enzymes produced not only by insects and plants but also by a wide group of microorganisms including fungi, bacteria, and archaea [43]. These enzymes break the cellulose into glucose and other fermentable sugar molecules. Exoglucanases (EC 3.2.1.91 and 3.2.1.176) gradually act on reducing and nonreducing ends of the cellulose to release cellobiose moieties, whereas endoglucanases (EC 3.2.1.4) arbitrarily cleave internal  $\beta$ -1,4-glucan linkages, while  $\beta$ -glucosidases (EC 3.2.1.21) reduce disaccharides to release glucose units [44]. Figure 5 represents activity of exoglucanase and endoglucanase enzymes in *A. brassicae* when treated with different concentrations of T1-T5. The results showed that exoglucanase enzyme activity was found to be decreased from 0.191 U/mL/h in the control (T1) to 0.002 U/mL/h at 200 ppm concentration of Tb-ZnO nanoparticles. Also, endoglucanase enzyme activity was found to be decreased from 0.048 U/mL/h in control (T1) to 0.01 U/mL/h at 200 ppm concentration of Tb-ZnO nanoparticles. The activity of both the enzymes was also found to be decreased after treatment with C-ZnO nanoparticles but the lowest concentration was observed in Tb-ZnO nanoparticles.

**3.8. Lipase Activity.** Activity of lipases with all the treatments (T2-T5) has been found to be decreased as compared to the control (T1). After titrimetric analysis, it was observed that in the control, the extracellular and intracellular lipase activities were 44.8 U/mL/h and 20.74 U/mL<sup>-1</sup>/h, respectively, whereas decrease in activity was found in treatment T4-T5 as the concentration of the treatment increased. Figure 6 represents the decrease in extracellular and intracellular lipases at different concentrations. Extracellular lipase activity of *A. brassicae* was reduced to 14.44 U/mL<sup>-1</sup>/h and 2.22 U/mL<sup>-1</sup>/h at C-ZnO nanoparticles (200 ppm) and Tb-ZnO nanoparticles (200 ppm), respectively, whereas intracellular activity was reduced to -3.14 U/mL<sup>-1</sup>/h and 7.03 U/mL<sup>-1</sup>/h at C- ZnO nanoparticles (200 ppm) and Tb-ZnO (200 ppm), respectively.

From the results, it was analysed that Tb-ZnO nanoparticles decrease the activity of cellulase enzymes and also lipase enzymes. These cellulase and lipase enzymes help the *A. brassicae* to enter inside the host tissue by hydrolysing cellulose and lipid units of the tissue. This retarded activity of these two enzymes will ultimately affect the pathogenicity of *A. brassicae* [45].

**3.9. Effect on Stress Enzymes.** External or internal stresses to the cell often resulted in build-up of reactive oxygen species (ROS), generating oxidative stress to the fungal cells. In response to this stress, fungi have evolved antioxidant mechanisms that have enzyme families that act on ROS, e.g., superoxide dismutase (SOD), catalases (CAT), peroxidases, and glutathione reductases [46]. The nanoparticle stress on the fungi has been reported by Zhang et al. The first protective enzyme is SOD against oxidative stress from superoxide radical anions. The by-product of SOD catalysed reaction or a product of extracellular stress which is H<sub>2</sub>O<sub>2</sub> is decomposed by catalase enzyme. These two enzymes play very important role in fungal defence mechanism in response to stress [47].

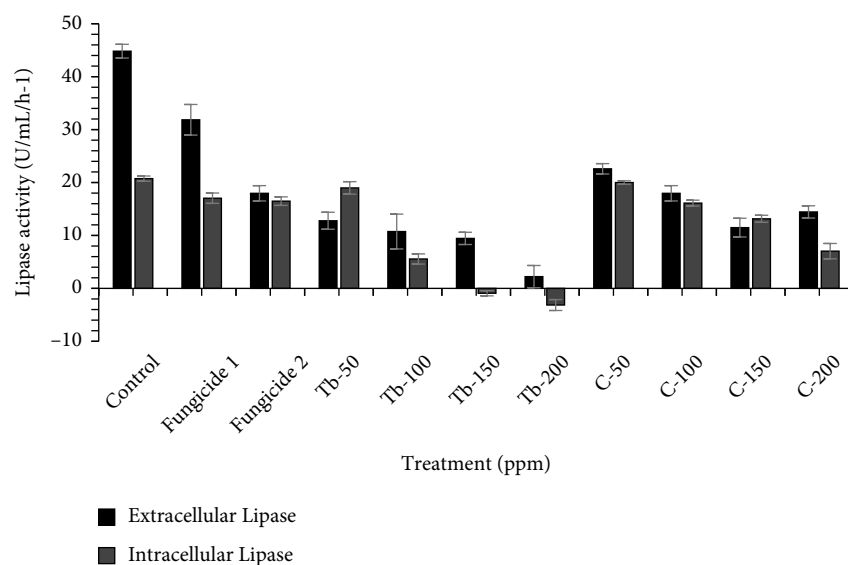


FIGURE 6: Extracellular and intracellular lipase activity of *Alternaria brassicae* treated with different concentrations of T1-T5. Bars are the means of three replicates and vertical line represents the standard error ( $\pm$ SE).

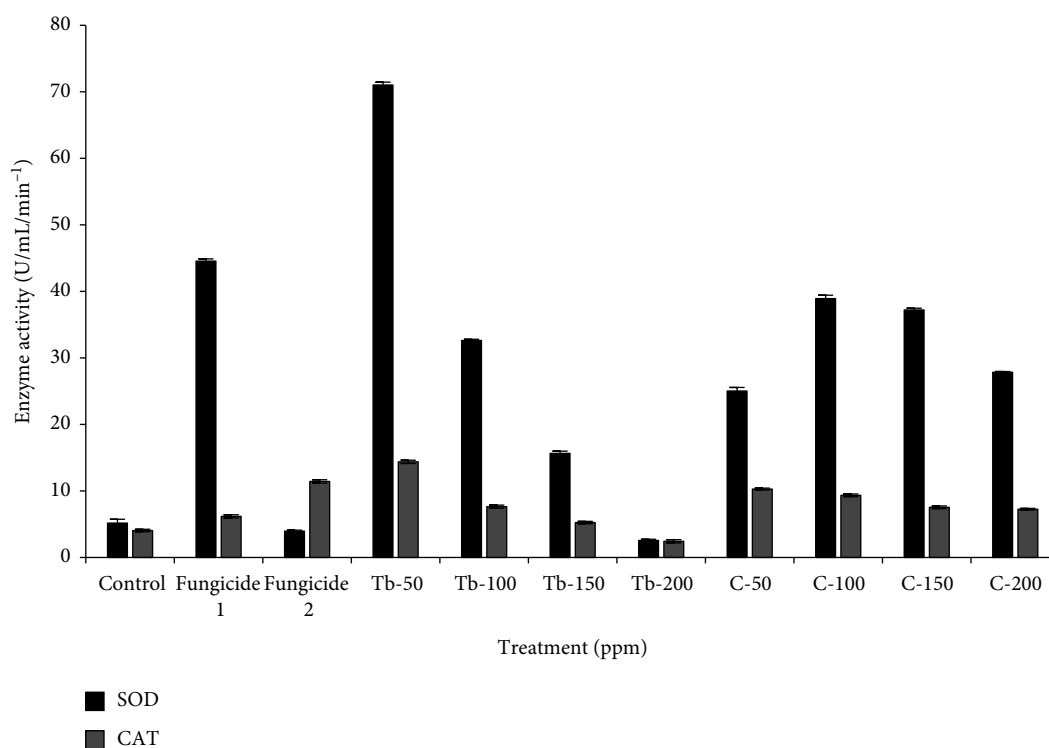


FIGURE 7: Effect on SOD and CAT enzymes in *A. brassicae* treated with different concentrations of T1-T5. Bars are the means of three replicates and vertical lines represent the standard error ( $\pm$ SE).

**3.9.1. Superoxide Dismutase and Catalase.** Figure 7 represents the effect of different concentrations of T1-T5 stress on *A. brassicae*. The increased activity of SOD and CAT enzymes represents the nanoparticles stress on the *A. brassicae*. As compared to the control, the activity of SOD and CAT activity was increased till Tb-ZnO nanoparticles

(50 ppm), whereas in C-ZnO nanoparticles, the activity was gradually increasing till C-ZnO nanoparticles 100 ppm, and after this concentration, the activity starts decreasing. Similarly, in case of Tb-ZnO nanoparticles after 50 ppm concentration, the activity of antioxidant enzymes decreased. These results also support the results of antifungal potential of Tb-



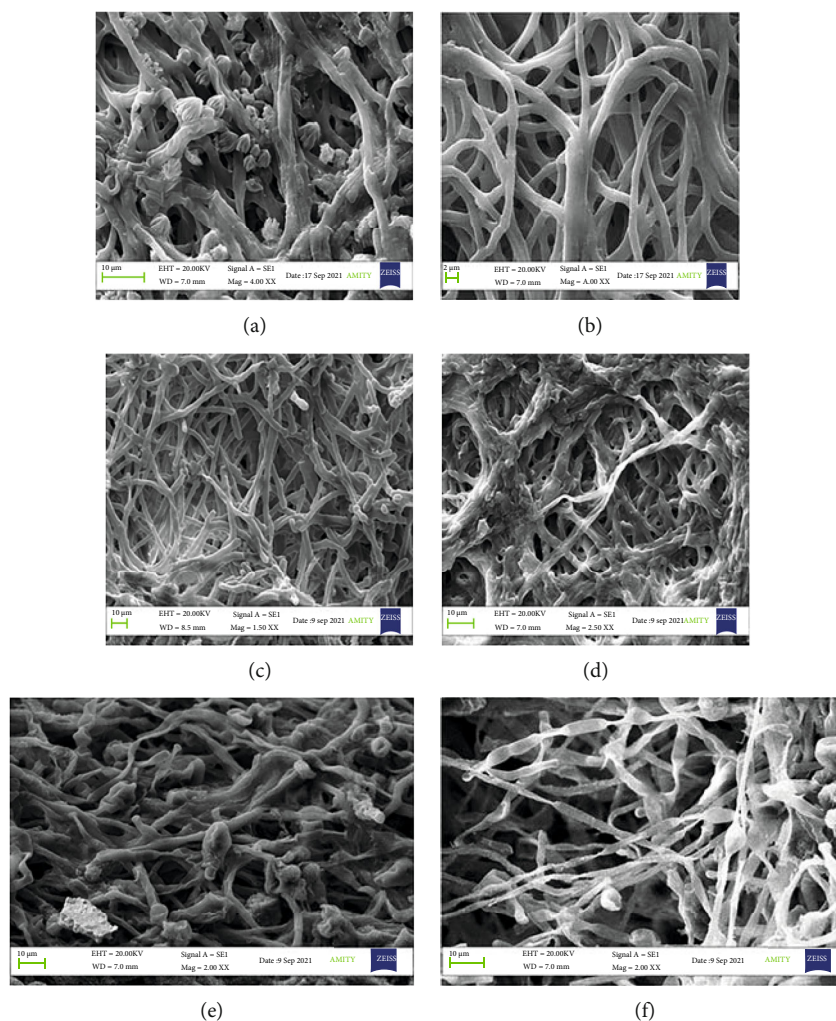


FIGURE 8: SEM images of treated *Alternaria brassicae* (a and b) control, (c) *Alternaria brassicae* treated with Mancozeb, (d) *Alternaria brassicae* treated with Propiconazole, (e) *Alternaria brassicae* treated with C-ZnO nanoparticles (200 ppm), and (f) *Alternaria brassicae* treated with Tb-ZnO nanoparticles (200 ppm).

ZnO nanoparticles against *A. brassicae*. The reason for their decreased activity might be the downregulation of SOD and CAT genes at increased concentration of nanoparticles [48].

**3.10. SEM Analysis of Treated *A. brassicae*.** Fungal cell walls are dynamic structures that are essential for cell viability, morphogenesis, and pathogenesis. The cell wall is the first point of contact between an antifungal molecule and a fungal cell. Fungal cell walls are composed mainly of 1,3- and 1,6- $\beta$ -glucans, chitin, and glycoproteins and aid in many functions, including providing cell rigidity and shape, metabolism, ion exchange, and interactions with host defence mechanisms. The mechanical strength of the fungal cell wall makes it a key structure in protection against a range of biotic and abiotic threats [49]. Cell wall of fungi has a complex structure and is primarily composed of polysaccharides. Cell wall plays an important role in defining the shape of the cell and protects the cell from external stress. To know the morphological changes in cell wall of fungus, *A. brassicae* was treated with ZnO NPs and SEM analysis has been employed. Figure 8 represents the mor-

phology of fungal spores and hyphae in treated and untreated culture of *A. brassicae*. Figures 8(a) and 8(b) show the presence of spores and smooth surfaces of hyphae, respectively, in the negative control (untreated), whereas Figures 8(c) and 8(d) are the positive controls showing only presence of abnormal hyphae but no spores. Figure 8(e) shows the SEM images of *A. brassicae* treated with C-ZnO nanoparticles (200 ppm) where the distorted, irregular fungal hyphae and spores can be seen. Figure 8(f) shows the irregular, deformed hyphae and absence of spores in SEM image of *A. brassicae* treated with Tb-ZnO NPs (200 ppm). Tb-ZnO nanoparticle-treated fungi indicate the extensive damage to the cell wall of hyphae which promotes the leakage of intracellular components and hyphal shrinkage. Similar results were also observed by different concentrations of copper oxide nanoparticles (CuO NPs) on “*Fusarium solani* (strain INECOL\_BM-04), *Neofusicoccum* sp. (strain INECOL\_BM-03), and *Fusarium oxysporum* (strain INECOL\_CBF-185).” They have also observed the irregular, deformed hyphae of fungi after treatment with green synthesized CuO NPs [50].

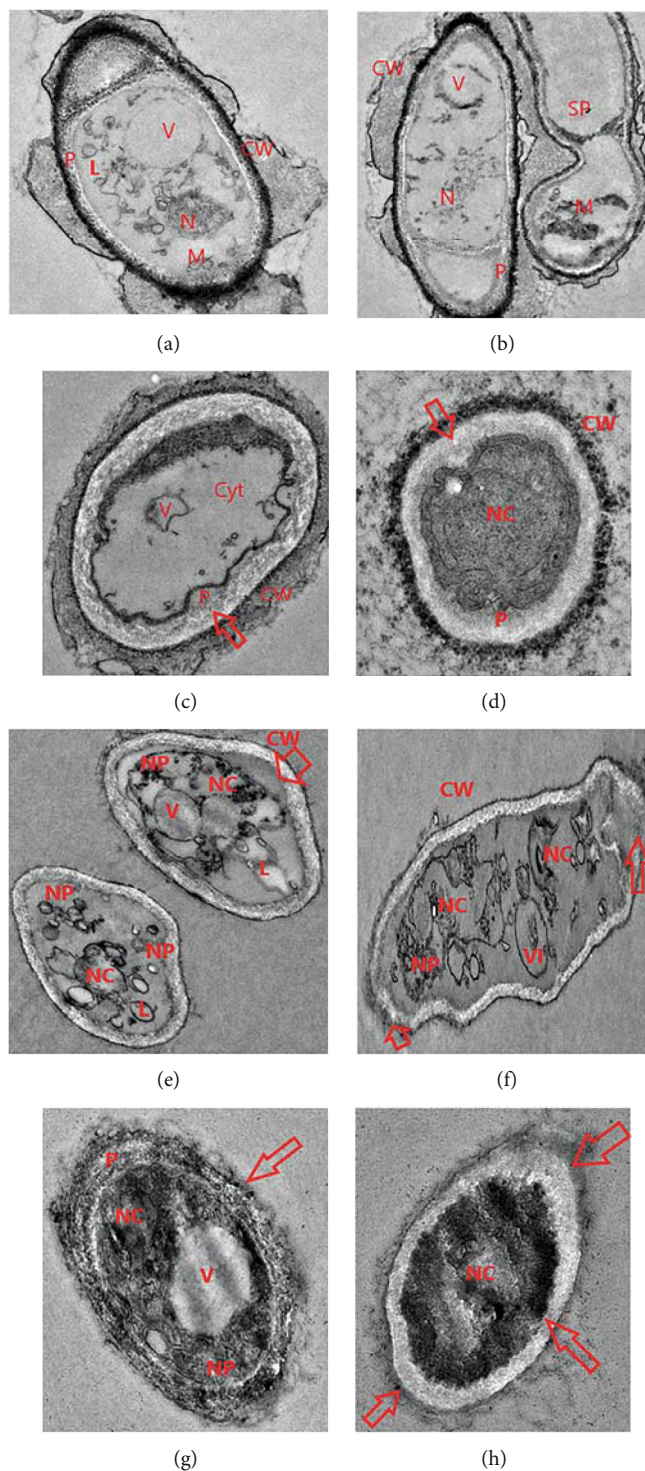


FIGURE 9: TEM images of treated *Alternaria brassicae*: (a and b) Control, (c) *Alternaria brassicae* treated with Mancozeb, (d) *Alternaria brassicae* treated with Propiconazole, (e and f) *Alternaria brassicae* treated with C-ZnO nanoparticles (200 ppm), and (g and h) *Alternaria brassicae* treated with Tb-ZnO nanoparticles (200 ppm). \*CW = cell wall; P = fungal plasmalemma; V = vacuoles; NP = nanoparticles; NC = distorted cytoplasmic content; N = nucleus; L = lipid droplets; VI = vesicular inclusions; M = mitochondria; SP = septa.

3.11. TEM Analysis of Treated *A. brassicae*. TEM analysis was done to analyse the ultrastructure of *A. brassicae* after treatment with the nanoparticles. TEM images confirmed the presence of regular inner and outer layers cell wall, fungal plasmalemma,

and cytoplasmic organelles in the negative control (Figures 9 (a) and 9(b)), whereas, in case of mancozeb (Figure 9(c)) and propiconazole (Figure 9(d)) treated *A. brassicae* (positive control) irregular fungal plasmalemma, deformed cell wall and

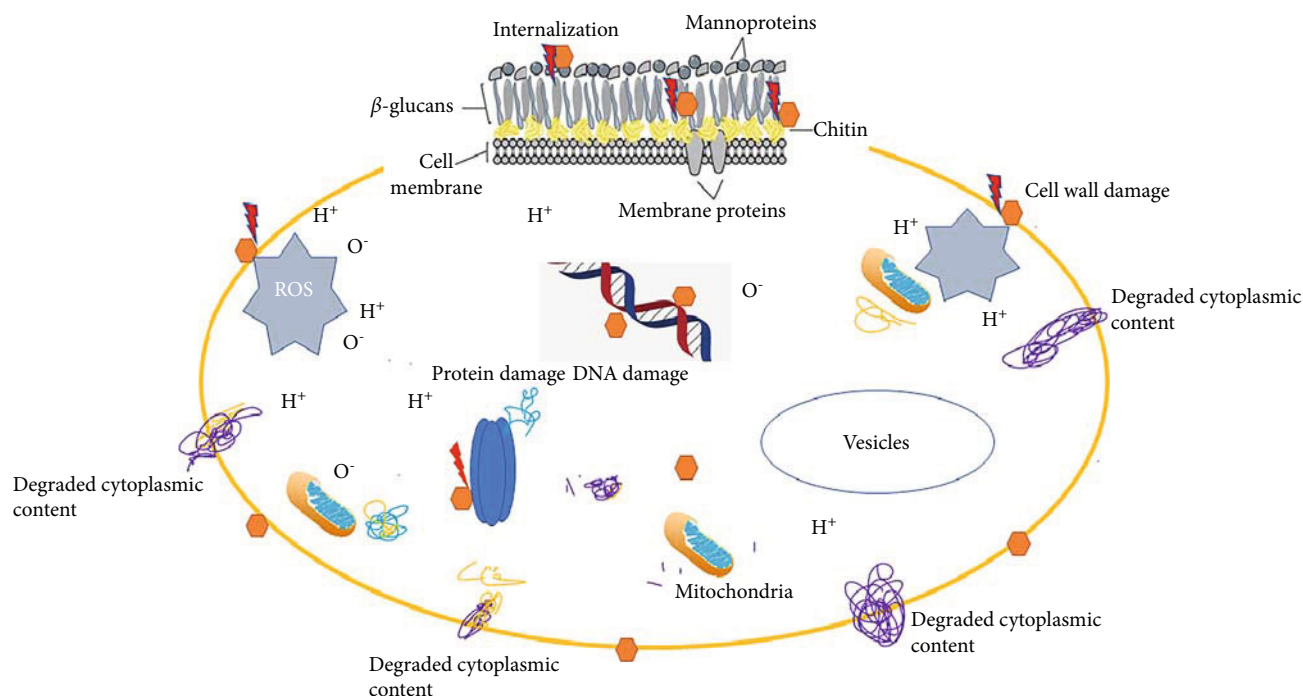


FIGURE 10: Possible mechanism of action of Tb-ZnO nanoparticles against *Alternaria brassicae*.

distorted cytoplasmic structures can be seen. The *A. brassicae* treated with C-ZnO nanoparticles (200 ppm) (Figures 9(e) and 9(f)) showed irregular plasmalemma, partially distorted cytoplasmic organelles including intracellular vesicles, aggregated glycogen granules, and vacuoles. The thinning of outer layer of cell wall resulted in partially damaged cell wall and leakage of cytoplasmic content (arrowed). However, the Tb-ZnO NPs (200 ppm) treated *A. brassicae* images showed the complete distortion of the inner and outer layers of cell wall, plasmalemma, and cytoplasmic organelles that results in leakage of cellular content (arrowed) outside the cell which is clearly visible in the images (Figures 9(g) and 9(h)). From these results, it can be assumed that Tb-ZnO NPs (200 ppm) inhibit the growth of *A. brassicae* by injuring cell wall and cell plasmalemma through direct physical interaction. Therefore, the growth inhibition of fungal hyphae and spores ascribed to toxic behaviour of Tb-ZnO NPs (200 ppm) which includes DNA damage, vacuolation, and distortion of organelles which are associated with the presence of nanoparticles inside the cell. The first step of fungal damage could be the absorption and internalization of nanoparticles that leads to the local damage to the cell wall following devastation of fungal plasmalemma and cytoplasmic content (DNA, protein, and lipid) which ultimately resulted into cell death [51, 52].

Ultrastructural studies done by TEM and SEM confirmed the deformation of cell wall in response to nanoparticles. Complete distortion of the cell wall and cytoplasmic content was found at 200 ppm concentration of Tb-ZnO NPs. Therefore, experimentally proposed mechanism of action of Tb-ZnO NPs on the basis of results obtained against *A. brassicae* includes (a) internalization of nanoparticles by fungal cell wall by damaging the cell wall, (b) development of stress inside the cell, (c) disintegration of essential

biomolecule synthetic machinery, and finally, (d) complete distortion of cell wall and release of degraded cytoplasmic content (Figure 10).

#### 4. Conclusion and Future Perspective

As per the literature, this is the first report suggesting antifungal mechanism of Tb-ZnO NPs by analysing their effect on protein, carbohydrate, chitin content and stress enzymes of fungal cell (*A. brassicae*). The present study suggests the significant antifungal potential of ZnO NPs synthesized from aqueous leaf extract of *Terminalia bellerica* against *A. brassicae* causative agent of leaf spot disease in *Brassica juncea*. Further, experiments are in progress such as optimization of nontoxic, fertile dose to the plants and in vivo study of leaf spot disease management in *B. juncea*.

ZnO has been declared safe for use by the FDA (The US food drug safety authority). Such synthesized nanoparticles can be used as nanofertilizers as zinc is an essential micronutrient for the plant growth. Application of optimized doses of ZnO NPs can suppress the disease as well as enhance the crop productivity. As food demand is increasing day by day, the yield of staple food crops is much low. So, it is need of the hour to commercialize metal nanoparticles for sustainable agriculture.

#### Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.



## Conflicts of Interest

The authors have no relevant financial or nonfinancial interests to disclose.

## Authors' Contributions

Shailja Dhiman and Arti Goel contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Shailja Dhiman. Ajit Varma has provided necessary lab equipment and chemicals required in the study. Ram Prasad has helped in the analysis and in writing the manuscript. The first draft of the manuscript was written by Shailja Dhiman and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Acknowledgments

We thank Dr. Sachin Kumar (Scientist) Dept. of Medical Oncology, Dr. BRA-IRCH, All India Institute of Medical Sciences, New Delhi, for their assistance for ultrastructural studies of nanoparticle-treated fungi.

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