

CS AND ZNO NANOPARTICLES AS FUNGICIDES
AGAINST POTATO FUNGAL PATHOGENS *ALTERNARIA*
SOLANI AND *FUSARIUM SOLANI*. MECHANISM
UNDERLINING THEIR ANTIFUNGAL ACTIVITY

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Abstract

Fusarium solani (*F. solani*) and *Alternaria solani* (*A. solani*) are among the most destructive pathogenic fungi affecting potatoes, causing significant crop losses in both field and post-harvest conditions. Current control relies heavily on synthetic fungicides which pose serious environmental and health risks. Therefore, there is a growing need to find efficient and environmentally friendly alternatives to combat fungal infections in potatoes. This study offers chitosan (CS) and zinc oxide (ZnO) nanoparticles (NPs) as potential fungicides against *A. solani* and *F. solani*. We evaluated their antifungal properties and elucidated the mechanism underlining their activity. Our results revealed

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that both CS and ZnO NPs exhibited pronounced antifungal activity against the two pathogenic strains by effectively inhibiting the mycelium growth. Furthermore, we discovered that the activity of the NPs involved the induction of oxidative stress in fungal cells and changes in the molecular organization of their membranes.

Key words: chitosan, zinc oxide, nanoparticles, *Alternaria solani*, *Fusarium solani*, fungicides

Introduction. Over a billion people depend on potatoes (*Solanum tuberosum* L.) for daily sustenance, making them one of the most important crops in the world [1]. However, fungus infections are becoming more common in potatoes, leading to significant yearly crop losses and poor crop quality. Among the numerous fungal pathogens affecting potatoes, *Alternaria solani* (*A. solani*) and *Fusarium solani* (*F. solani*) stand out as the most damaging ones. *A. solani* is the primary pathogen causing an early blight on potatoes, ranking as one of the most dangerous potato diseases worldwide [2]. *Fusarium solani*, on the other hand, causes roots and seed decay in tubers during storage, resulting in extreme harvest losses and mycotoxins contamination [3]. In efforts to control fungal infections in potatoes during farming and post-harvest stages agriculture heavily relies on synthetic fungicides. However, this practice poses a grave hazard for humans and other non-targeted organisms, depending on fungicide toxicity, contamination degree, and exposure duration [4]. Moreover, the widespread and frequent application of synthetic fungicides leads to the emergence of fungicide-resistant pathogen populations, as reported for potato isolates of *A. solani* [5]. Therefore, in the face of combating potato fungal infections, the pressing need for fungicide alternatives against *F. solani* and *A. solani* continuously arises. These alternatives should effectively prevent fungal development while guaranteeing the safety of humans and the environment by meeting crucial criteria for bio- and eco-compatibility.

Organic and inorganic nanoparticles have a promising future as fungicides in agriculture as they are recognized as eco-friendly materials. One organic compound that is particularly cutting-edge for nanoparticle formulation is chitosan (CS). CS is a linear polysaccharide possessing desirable biodegradability and biocompatibility [6]. The utilization of CS in agriculture has many advantages over other polymers as it possesses an inherent antifungal activity due to its polycationic nature without the need for any chemical modification [7]. Inorganic zinc oxide (ZnO) is a familiar compound in agriculture. Its bulk form is often added to macronutrient fertilizers that provide plants with essential Zn [8]. On the other hand, ZnO nanoparticles have shown an exceptional inhibitory effect on the growth of pathogenic fungi. It was discovered that their efficacy was size-dependent and through the realization of metal Zn ions, caused a severe stress response in fungi [9].

Considering the promising antifungal applications of chitosan (CS) and zinc oxide (ZnO) nanomaterials, this study aimed to present newly synthesized CS

and ZnO nanoparticles that could offer effective control over fungal infections in potatoes. This research explores, for the first time, the antifungal activities of these nanoparticles against *A. solani* and *F. solani* under in vitro conditions. Furthermore, the study delves into the molecular mechanisms underlying their antifungal activities by measuring several oxidative stress markers and examining quantitative alterations in the molecular organization of fungal membranes. The findings of this study provide a valuable basis for the future application of these nanoparticles as alternatives to conventional fungicides.

Materials and methods. Materials. Fungal strains. *Fusarium solani* (CBS 260.54) and *Alternaria solani* (CBS 106.21) are part of the Mycological collection of the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences. Both fungal strains were preserved at -80°C in the Microbank system (Prolab Diagnostics, Richmond Hill, Canada). Upon experiments, the conidiospores were grown on Potato dextrose agar (PDA) at 28°C for 7 days.

Nanoparticles. All chemicals utilized in the production of the CS and ZnO NPs were purchased from Sigma-Aldrich and were of analytical grade, used without further purification. CS NPs were prepared from low molecular weight chitosan (1.0 g, M. wt 112 000, deacetylation 85%) according to the method described in [11]. The ZnO NPs were synthesized from zinc acetate dihydrate (99.5%) as a colloidal suspension using a sol-gel technique by the method described in [10].

Fluorescent dyes and kits for oxidative stress biomarkers assay. All fluorescent dyes, chemicals, and assay kits were purchased from Sigma Aldrich unless stated otherwise. Laurdan (6-dodecanoyl-N, N-dimethyl-2-naphthylamine) fluorescent probe was used for membrane order measurements. Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) was used for cell viability assays. The content of carbonyl groups in oxygen-modified proteins was determined by the Protein carbonyl content (PCC) kit. The level of lipid peroxidation was measured by a lipid peroxidation (MDA) assay kit measuring the amount of malonaldehyde.

Methods. NPs dispersion preparation. Stock solutions with a concentration of 1 mg/ml of the CS and ZnO NPs were prepared in 0.2% dimethyl sulphoxide (DMSO).

Microdilution method. The NP dispersions were diluted in sterile 96-well plates with potato dextrose broth (PDB) in a progression concentration – 0.05; 0.10; 0.15; 0.20; 0.25; 0.30; 0.35 and 0.40 $\mu\text{g}/\text{ml}$. Ten μl fungal spore suspension at a concentration of 1×10^8 conidia/ml was inoculated in each well and subsequently, 30 μl of resazurin (0.02%) was added. 0.1% nystatin was used as the positive control and 0.2% DMSO was used as the negative control. The plates were then incubated for 24, 48, 72, and 144 h at 28°C . The experiments including the control variants were carried out under the same conditions in triplicates with four repetitions per experiment. The effect of tested NPs on fungal growth was evaluated by visual inspection.

Submerged cultivation and analytical assay. For inoculum preparation, submerged cultivation was performed for 24 h at 25 °C in 500 ml flasks. Submerged cultivation was performed for 72 h at 25 °C in 200 ml flasks. Thirty ml PDB and three ml spore suspension at a concentration of 2×10^8 spores/ml were used. The NPs were added at the 48th hour of incubation in two aliquot concentrations of 0.5 and 1 mg/ml. The control variants were also carried out under the same experimental conditions. The cultivation was performed for up to 72 h at 25 °C.

The superoxide dismutase (SOD) activity was determined by the method developed by Beauchamp and Fridovich. The activity of SOD was quantified as Units/mg protein. One unit of SOD activity represents the amount of enzyme protein required to inhibit the NBT (NitroBlueTetrazolium) reduction rate (A560) by 50% at pH 7.8 and 30 °C. Standard bovine serum was used to determine the protein amount. The content of carbonyl groups in oxygen-modified proteins was determined by the Protein carbonyl content (PCC) kit. The determination involved derivatization with 2,4-dinitrophenylhydrazine (DNPH), resulting in the formation of stable dinitrophenyl (DNP) hydrazone adducts. The measurement was carried out spectrophotometrically at 375 nm. The level of lipid peroxidation was measured by a lipid peroxidation (MDA) assay kit measuring the amount of malonaldehyde. The determination involved the reaction between malondialdehyde (MDA) with thiobarbituric acid (TBA), producing a colorimetric product proportional to the amount of the present MDA. The measurement was conducted spectrophotometrically at a wavelength of 532 nm. All experiments were performed in triplicate with four repetitions per experiment.

Laurdan fluorescent spectroscopy. Both fungal strains were suspended in 10 ml 10 mM Hepes. Subsequently, they were centrifuged three times for 10 min at 1000 rpm. The resulting precipitates were resuspended in 1 ml Hepes. Fifty μ l of Laurdan from a stock of 10 mg/ml in chloroform/methanol (9:1), was placed under a stream of oxygen-free dry nitrogen. The obtained dried Laurdan film was hydrated in 500 μ l of 0.2% DMSO. Two μ l of the prepared Laurdan solution was added to both fungal suspensions. The steady-state fluorescence was measured by a spectrofluorometer Jasco at a room temperature of 22 °C. One ml of fungal suspension with a concentration of 1×10^5 conidia/ml was added in a quartz cuvette. The NPs were added to the fungal suspensions in two aliquot concentrations of 0.5 and 1 mg/ml. Laurdan was excited at 355 nm and the emission spectra were recorded from 390 to 600 nm. All experiments were triplicated with four repetitions per experiment. The obtained spectra from the measurements were used to calculate the general polarization (GP) values as follows:

$$GP = (I_{440} - I_{490}) / (I_{440} + I_{490}).$$

I_{440} and I_{490} are the emission intensities at 440 and 490 nm. Theoretically, the GP values can range from -1.0 (being least ordered) to $+1.0$ (being most ordered) [12].

Statistical analysis. The statistical calculations were performed using OriginPro9.0. The experimental data were analyzed with one-way ANOVA. Each p -value can be considered statistically significant at $p < 0.05$.

Results. Nanoparticles characteristics. Several advanced techniques were employed for the characterization of the prepared NPs, as detailed in the references [10,11]. Dynamic light scattering (DLS) was utilized to measure the size distribution and zeta potentials of the NPs, using Zetasizer nano-ZS (Malvern Instruments Ltd, UK). ZnO NPs exhibited a smaller average size of 48 ± 3 nm, compared to the CS NPs with a size of 54 ± 4 nm. Zeta potential values were $+23 \pm 1$ mV for ZnO NPs and $+37 \pm 2$ mV for CS NPs. These high positive zeta potential values indicate a cationic surface nature, which results in strong repulsion between the NPs, preventing agglomeration and maintaining a stable colloidal suspension. Transmission electron microscopy (TEM) was used for the morphological visualization of the NPs. The TEM images showed spherical shapes for both CS NPs [11] and ZnO NPs [10]. The X-ray diffraction (XRD) patterns of the prepared NPs were also analyzed. ZnO NPs showed peaks assigned to the hexagonal phase structure of ZnO [10]. In contrast, the XRD pattern for the CS NPs showed two peaks corresponding to the diffraction peaks of chitosan [11]. The obtained XRD patterns confirmed the crystalline structure of the ZnO NPs and the semicrystalline structure of the CS NPs.

Antifungal activity. Both CS and ZnO NPs exhibited significant inhibitory effects against both fungal strains. CS NPs inhibited the growth of *A. solani* at a concentration of 0.4 mg/ml. Conversely, for *F. solani*, CS NPs exhibited inhibitory activity at a lower concentration of 0.3 mg/ml. ZnO NPs, on the other hand, demonstrated effective antifungal activity against both fungal strains at the highest tested concentration of 0.4 mg/ml.

Analytical assay. In both fungal strains, the SOD activity levels were increased following treatment with the CS NPs in a noticeable dose-dependent manner. In *A. solani* SOD level induced by 1 mg/ml of the CS NPs was nearly 13 times higher than the control (Fig. 1A). In *F. solani*, however, the higher concentration of the CS NPs showed only half of the potency compared to *A. solani* (Fig. 1B). ZnO NPs increased the SOD activity in *A. solani* albeit to a lesser extent compared to CS NPs with almost equal elevated levels at both tested concentrations (Fig. 1A). In *F. solani*, however, ZnO NPs did not show any impact on the SOD activity (Fig. 1B).

In the presence of the NPs, the PCC levels increased compared to their respective controls (Fig. 2). CS NPs demonstrated a dose-dependent increase in PCC levels in both fungal strains. Similar to the SOD activity, the effectiveness of 1 mg/ml of CS NPs on the PCC levels was twice as high in *F. solani* compared to *A. solani* (Fig. 2A, B). One mg/ml of ZnO NPs induced the same PCC levels in both fungal strains. However, the effect at the lower concentration of 0.5 mg/mg in *A. solani* was not significantly different from the control (Fig. 2A).

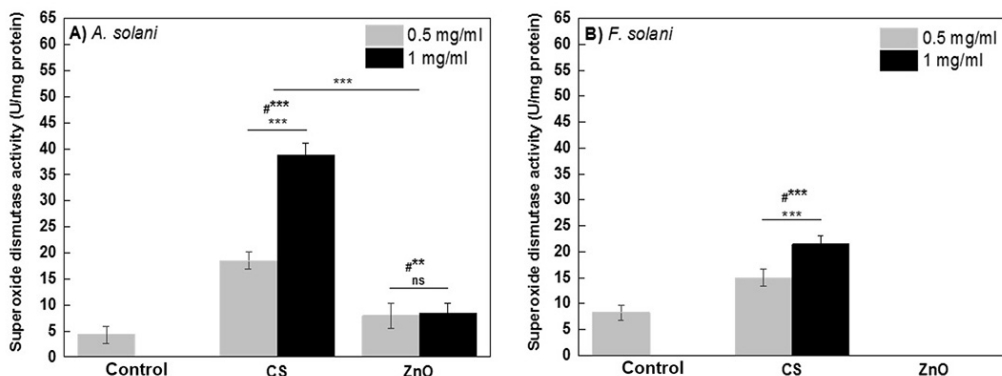


Fig. 1. Levels of superoxide dismutase activity (SOD) in U/mg protein depending on the type of NPs at concentrations of 0.5 mg/ml and 1 mg/ml. A) SOD levels in *A. solani* – Control is 4.25 U/mg protein. B) SOD levels in *F. solani* – Control is 8.26 U/mg protein. The standard deviations (SD) of SOD levels are obtained from three independent experiments with four measurements per sample. Statistical significance is denoted as follows: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. Comparison between the SOD levels induced by the NPs and the control is indicated with # as follows: **** $p < 0.001$, *** $p < 0.01$, #* $p < 0.05$. The designation “ns” (not significant) indicates statistically insignificant differences

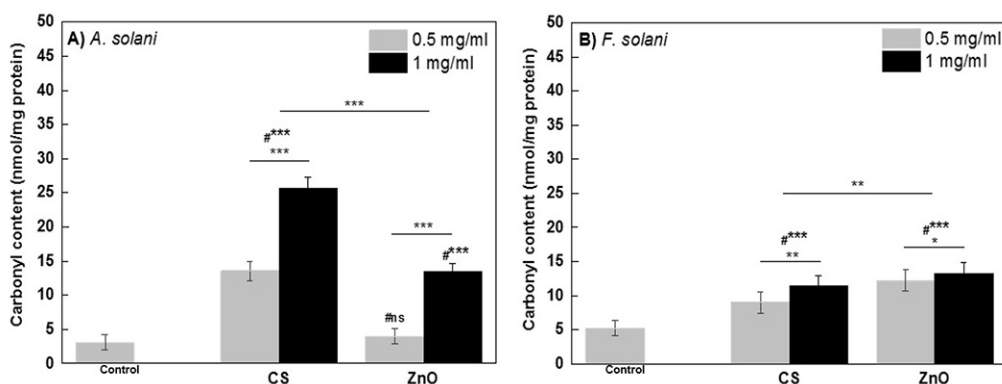


Fig. 2. Levels of protein carbonyl content (PCC) in nmol/mg protein, depending on the type of NPs at concentrations of 0.5 mg/ml and 1 mg/ml. A) PCC levels in *F. solani* – Control is 3.089 nmol/mg protein. B) PCC levels in *A. solani* – Control is 5.2 nmol/mg protein. SD of SOD levels are obtained from three independent experiments with four measurements per sample. Statistical significance is denoted as follows: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. Comparison between the PCC levels induced by the NPs and the control is indicated with # as follows: **** $p < 0.001$, *** $p < 0.01$, #* $p < 0.05$. The designation “ns” (not significant) indicates statistically insignificant differences

Following treatment with the NPs, the MDA levels increased in a noticeable dose-dependent manner (Fig. 3). In both fungal strains 1 mg/ml of CS NPs induced the highest elevated MDA levels. Conversely, ZnO NPs had a nearly similar effect on both fungal strains, resulting in comparable levels of MDA.

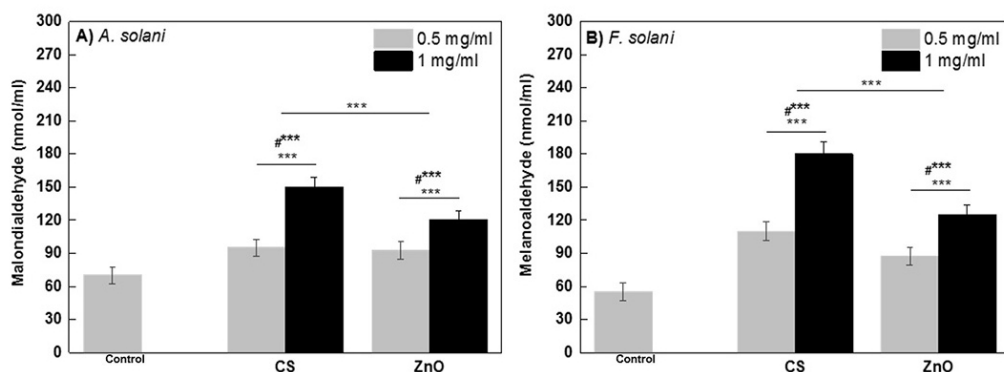


Fig. 3. Levels of malondialdehyde (MDA) in nmol/ml, depending on the types of NPs at concentrations of 0.5 mg/ml and 1 mg/ml. A) MDA levels in *A. solani* – Control is 70 nmol/ml; B) MDA levels in *F. solani* – Control is 55 nmol/ml. SD of MDA levels are obtained from three independent experiments with four measurements per sample. Statistical significance is denoted as follows: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. Comparison between the MDA levels induced by the NPs and the control is indicated with # as follows: #*** $p < 0.001$, #** $p < 0.01$, #* $p < 0.05$

Membrane order alternations. At the lower concentration of 0.5 mg/ml, CS NPs decreased the GP values in both fungal strains. However, at the highest concentration of 1 mg/ml, the GP value in *F. solani* was higher than the control, whereas, in *A. solani*, it was lower than the control, indicating opposite effects depending on the fungal strain.

In *F. solani* a considerable increase in GP value was observed by 0.5 mg/ml of ZnO NPs. The difference between the GP values of 0.5 and 1 mg/ml was slight yet noticeable. In *A. solani* ZnO NPs exhibited a prominent dose-dependent effect with an exponential increase in GP values.

Delta GP (Δ GP) represents the difference between the highest GP values (at the highest concentration of the NPs) and the GP value of the control. Δ GP serves as a graphical representation of the total quantitative changes in membrane organization after treatment with the NPs. ZnO NPs exhibited a conspicuous ordering effect on both fungal strains, with the most significant quantitative order changes observed in *F. solani*. These changes exceeded those in *A. solani* by more than 5 times. Conversely, CS NPs exhibited opposite effects on both fungal strains. While membranes of *F. solani* exhibited a slight order increase following treatment with the ZnO NPs, membranes of *A. solani* experienced a decrease in molecular order, suggesting a fluidizing effect on the fungal membrane.

Discussion. The presented CS and ZnO NPs in this study demonstrated efficient antifungal activity against *A. solani* and *F. solani*. An effective inhibition of mycelium growth was observed at 0.4 mg/ml. The antifungal activity of the NPs is directly attributed to the combination of their characteristics, particularly size and surface charge. The average size of the examined NPs is 48 nm for

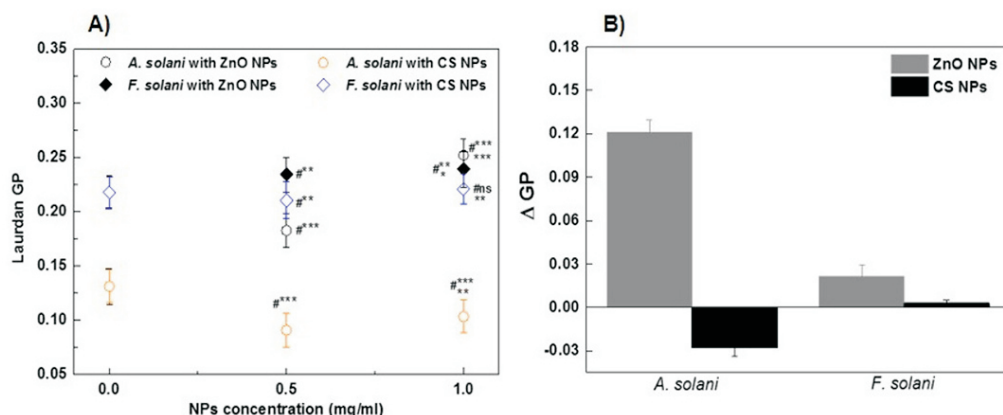


Fig. 4. Alterations in the fungal membrane order by Laurdan fluorescent spectroscopy. A) Laurdan GP of *A. solani* and *F. solani* in dependence of the NPs concentrations in two aliquote concentrations (0.5 and 1 mg/ml) at room temperature (22 °C). The standard deviations (SD) are obtained from three independent experiments with four measurements per sample. Statistical significance between the GP values of 0.5 mg/ml NPs and 1 mg/ml NPs is denoted as follows: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. Comparison between the GP values at both concentrations of the NPs and the control (without NPs) is indicated with # as follows: #*** $p < 0.001$, #** $p < 0.01$. The designation “#ns” (not significant) indicates statistically insignificant differences from the control. B) Laurdan ΔGP in both fungal strains. $\Delta GP = GP (0.05 \text{ mg/ml}) - GP (0 \text{ mg/ml})$

ZnO NPs and 54 nm for CS NPs, indicating a narrow size distribution profile. Smaller (40 nm) NPs are shown to exert greater toxic effects than larger ones (around 100 nm) [13], because of their higher surface area-to-volume ratio, which facilitates stronger interactions with the fungal surfaces and promotes stronger antifungal activity [14]. The surface charge is another key parameter contributing to the antifungal potency of the NPs. The average zeta potential values of the examined NPs are + 23 mV for ZnO NPs, and + 37 mV for CS NPs, indicating a cationic surface charge. The positive surface charge of the NPs contributes to their interaction with the negatively charged microbial cell surface, leading to an impairment of cell activity [15].

Both CS and ZnO NPs induced significant levels of oxidative stress in both fungal strains, highlighting their excellent pro-oxidative potential. Following treatment with the NPs elevated levels of different stress biomarkers were detected such as activated superoxide dismutase, protein carbonyl content, and malondialdehyde, a marker for lipid peroxidation. Notably, treatment with 1 mg/ml of the CS NPs demonstrated the most outstanding pro-oxidative effect in both strains. However, the exact mechanism of how CS NPs can generate oxidative stress remains unclear. It was previously proposed that CS-induced cellular ROS burst is directly associated with the rapid oxidation of fatty acids in the cell membranes. Increased membrane oxidation ultimately leads to plasma membrane permeabi-

lization and is perhaps responsible for the antifungal effect of CS [16]. On the other hand, ZnO NPs are known for their prominent pro-oxidative activities. Under ultraviolet and visible light ZnO transits electrons (e^-) from the valence band, leaving positively charged holes (H^+). Consequently, the surface of ZnO NPs becomes highly reactive and can interact with water and oxygen generating high levels of ROS species [17]. Moreover, ZnO NPs can release reactive Zn^{2+} ions causing impairment of the electron transport system and denaturation of cellular proteins [18].

This study further revealed that the antifungal effect of the NPs is associated with direct interactions with the fungal cell membranes and subsequent molecular alternations in the membrane bilayer. The quantitative membrane order changes in the fungal membranes were measured by Laurdan fluorescent spectroscopy. The method is based on the phenomena of dipolar relaxation, giving information about the concentration and dynamics of free water molecules associating with the glycerol backbone of the membrane lipids [19]. The general polarization (GP) allows us to quantify the molecular order changer in the bilayer upon interactions with the tested NPs. Our results showed that the membranes of *A. solani* were more susceptible to the activity of the NPs with the greatest order changes. Moreover, the impact of ZnO NPs on membrane ordering was notably more pronounced when compared to CS NPs. The positive charge of NPs enables them to interact with negatively charged phospholipid components of fungi membranes [20]. Consequently, electrostatic attraction emerges leading to the adhesion of the NPs on the fungal membrane surfaces. Moreover, the small sizes of the NPs can facilitate their penetration into the bilayer causing dehydration of the membrane by substitution of the water molecules around the glycerol backbone of the lipids. This explains the increased values of GP of the fungal membrane order after treatment with the NPs.

Conclusions. The present study demonstrates that both CS and ZnO NPs exhibit efficient antifungal potency against phytopathogenic fungi *Fusarium solani* and *Alternaria solani*. This activity of the NPs involves a complex mechanism, including the induction of oxidative stress and membrane order alternations. The NPs exhibited significant pro-oxidative activities by generating high levels of ROS causing activation of the cellular antioxidant system, oxidation of cellular proteins, and induction of lipid peroxidation. Furthermore, we revealed that the activity of the NPs involved interactions with the fungal cell membranes and consequent alternations in the membrane organization. The findings in this study suggest that the CS and ZnO NPs could serve as effective alternatives to fungicides, offering promising control over fungal diseases in potatoes.

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