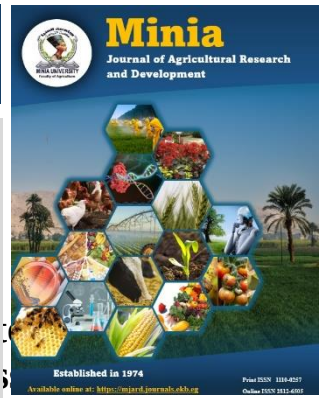
Minia J. of Agric. Res. & Develop., Minia Univ., Vol. 45 (4): 1019 - 1048, 2025

Minia Journal of Agricultural Research and Development

Journal homepage & Available online at:

https://mjard.journals.ekb.eg



Improving *Trichoderma harzianum's* Efficiency in Combatting Late Wilt Disease in Maize with Chitosan and Chitosan Nanoparticles

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Received: 6 Nov. 2025 Accepted: 12 Nov. 2025

ABSTRACT

In developing nations, maize *(Zea mays* L.) is a vital grain crop. *Magnaporthiopsis maydis* is the fungus that causes maize late wilt, it is considered a serious fungal disease in Egypt. El-Minia isolate-$_2$ was the most virulent one on Boushy cv. maize in vivo. *M. maydis* growth on PDA plates in vitro was strongly reduced by chitosan and chitosan nanoparticles (chitosan-NPs) at 50, 100, 150, and 200 ppm. *Trichoderma harzianum* mycelium growth remains strongly unaffected by chitosan concentrations from 50 to 150 ppm, while it decreases significantly at concentrations up to 200 ppm. Transmission electron microscopy (TEM) showed that *M. maydis* and *T. harzianum* mycelium cell membranes are structurally altered by chitosan and chitosan NPs. TEM images of ultrathin slices showed *M. maydis* mycelium with partial cell -wall lysis and membrane shrinkage. Chitosan NPs damaged fungal cell walls and membranes, killing cells, while the non-treated control had well-defined organelles. *T. harzianum* and chitosan nanoparticles at tested concentrations (individually or in combinations) improved plant resistance to pathogen and significantly reduced the disease incidance. Furthermore, these treatments increased β-1,3-glucanase, peroxidase, and catalase enzyme activities in maize leaves compared to both infected and uninfected control. In the 2024 growing season, *T. harzianum* and chitosan NPs at 150 ppm were the most effective treatment for increasing enzyme activities, mitigating late wilt disease, and improving maize growth parameters and yield components at Gemmieza and Malawy Research Stations.

Keywords: late wilt disease, *Magnaporthiopsis maydis*, *Trichoderma harzianum*, Chitosan, Chitosan nanoparticles, Enzyme activities.

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BACKGROUND

Maize (*Zea mays* L.) is the third most important cereal crop worldwide (Family *Poaceae*), ranking behind wheat and rice. In Egypt, it is an essential source of food for both humans and animals, and poultry feed, and used as a raw ingredient for commercial products like starch and oil. Additionally, maize can be harvested while still green to be made into silage, which is used as winter feed for farm animals (**Grote *et al.*, 2021; Sanodiya & Gupta, 2023**).

The global area cultivated with corn spans approximately 200 million hectares across various countries **(Erenstein *et al*., 2022)**. In Egypt, the planted area under corn reached 2.4 million feddans in 2022, resulting in a total production of 7.7 million tons. This local maize output is insufficient to meet the high demand, especially for yellow grains, prompting Egypt to import corn to bridge the gap between production and consumption **(Anonymous, 2022; Adly *et al*., 2024)**. Additionally, the Egyptian government has recently introduced several policies and programs aimed to increasing maize production. These initiatives focus on utilizing high-yielding hybrids and enhancing agricultural practices (**Kotb & Mansour, 2012; Ali & Abdelaal, 2020)**. Moreover, funding has been allocated for research and extension services, improvements to irrigation infrastructure, and subsidies for essential inputs such as seeds and fertilizers **(Ismail *et al.*, 2024)**.

Late wilt is a serious disease caused by the fungus *Magnaporthiopsis maydis*, and it is one of the biggest problems for growing maize in Egypt. As a new genius in the *Magnaporthaceae* (Ascomycota) family, *Magnaporthiopsis* was introduced by Luo and Zhang in 2013. Ultimately, *Magnaporthiopsis maydis* replaces *C. maydis* in the classification (Klaubauf et al., 2014) synonymous: *Harpophora maydis* **(Games, 2000),** and one of the most major diseases affecting maize in Egypt is *Cephalosporium maydis* (**Samra *et al*., 1963; Sabet *et al*., 1966; Ali, 2000 and Saleh & Leslie, 2004)**. This fungus invades the plant's xylem, leading to late wilting, as noticed by **Sabet *et al*. (1961 and 1970)**. The disease results in significant production and productivity losses worldwide, impacting several nations **(El-Naggar & Sabry, 2011; Ortiz-Bustos *et al*., 2015)**. The extent of damage caused by *M. maydis* to hybrid Egyptian maize depends on the cultivar's susceptibility and the timing of symptom onset (**El-Shehawy *et al*., 2014; El-Hosary *et al*., 2015)**. There is a negative correlation between disease incidence and grain yield, with potential yield losses ranging from 40% to 70% **(El-Naggar *et al*., 2015; Hassan *et al*., 2022)**.

The economic significance of late wilt disease (LWD) in maize underscores the necessity for effective management strategies to manage this disease or, at the very least, to reduce its detrimental influence on the survival of plants and grain yield **(El-Naggar *et al*., 2015; Farahat *et al*., 2020)**. Emerging strategies, including biological control **(El-Shahawy & El-Sayed, 2018; El-Shabrawy & Shehata, 2018)** and induction of host resistance through the application of organic acids, organic salts, and essential oils **(Abdel-Kader *et al*., 2022),** represent sustainable alternatives to minimize the excessive reliance on chemical fungicides, which can contribute to environmental contamination, increased health risks, and the emergence of antimicrobial-resistant organisms **(Degani & Cernica, 2014; El-Moghazy *et al*., 2017)**.

Trichoderma species, including *T. harzianum, T. hamatum, T. longibrachiatum, T. kongsi, T. viride, T. polypore*, and *T. asperellum*, have been extensively studied and employed as effective biological control agents for the suppression and management of a wide range of fungal plant diseases due to their mycoparasitic abilities against various phytopathogenic fungi (**Kubicek *et al*., 2019; Di Marco *et al*., 2022)**. Additionally, *Trichoderma species* they are known to produce a wide range of bioactive compounds, including cell wall-degrading enzymes and diverse secondary metabolites involved in biocontrol and plant growth promotion. They can greatly enhance crop tolerance, reduce plant diseases, and promote plant growth due to their antagonistic characteristics against nematodes and plant-pathogenic fungi (**Druzhinina *et al*., 2018; Kubicek *et al*., 2019**).

Chitosan is a prevalent natural polymer (poly (1,4)-2-amino-2-deoxy-β-D glucose), which is a polymer of glucosamine **(Negm *et al*., 2020**). It is produced through the alkaline deacetylation of chitin, a substance derived from the exoskeleton of crustaceans (**Hadwiger and Beckman**, **1980; Priyadarshi & Rhim, 2020**).

Scientifically, chitosan has garnered considerable interest due to its versatile applications in plant protection and growth stimulation, attributed to its excellent biodegradability, non-toxicity, and bioactive properties. **(Chandra *et al*., 2015, Xing *et al*., 2015; Mahmood *et al*., 2017)**. Additionally, chitosan possesses unique antimicrobial properties **(Silva *et al*., 2014; El Gamal *et al*., 2016)**. Its biocompatibility, biodegradability, and low toxicity enhance its effectiveness as a preventive plant defense mechanism against pathogen attacks. Moreover, chemically modifiable functional groups in chitosan open numerous potential applications (**Mahmood *et al*., 2017**). Research indicates that the capability of chitosan is significantly enhanced when used in nanoparticle form, making chitosan-based nanomaterials highly attractive as carriers **(Osuwa & Anusionwu, 2011; Chandra *et al*., 2015)**. Engineered nanoparticles exhibit enhanced chemical and physical properties due to their nanoscale size, distribution, and shape compared to their bulk counterparts **(Bakshi *et al*., 2014; Elizabeth *et al*., 2019; El-Saadony *et al*., 2021).**

The purpose of this study is to evaluate the effectiveness of the chemical inducer chitosan, especially its nanoform, and the bioagent *T. harzianum*, combined, to resist LWD in maize.

MATERIAL AND METHODS

Isolation and identification of the pathogen

Maize plants showing typical symptoms of late wilt disease were collected from open field conditions in the El-Minia, El-Beheira, El-Giza, and Kafr El-Sheikh Governorates of Egypt. The collected specimens were rinsed with tap water, blotted dry between sterilized filter papers, surface-sterilized for 2 minutes in 2% sodium hypochlorite solution, and then thoroughly rinsed several times with sterilized distilled water. Pieces of the plants were cultivated for three to seven days at 26 ± 2°C on potato dextrose agar medium (**Sabet *et al.* 1970).** The hyphal tip approach was employed to purify the cultures, which were subsequently examined under a microscope and kept at 4°C on PDA slants for advanced studies. The pathogenic isolate was identified as *Cephalosporium maydis* (*Magnaporthiopsis maydis*) as described by **Sabet *et al*. (1966)**

Antagonistic microorganisms

The Biolog-System technique was used to identify the microbial bioagent *T. harzianum*, which was obtained from the earlier study by **Khalifa (2016)** at the Identification of Microorganisms Unit, Plant Pathology Research Institute, A.R.C., Giza, Egypt.

Maize grains

Grains of the maize cultivar Boushy, characterized by its susceptibility, were procured from the Maize and Sugar Crop Diseases Research Department, Plant Pathology Research Institute, ARC, Giza, Egypt.

Pathogenicity test

The experiment was conducted in a controlled greenhouse environment of the Maize and Sugar Crop Diseases Research Department, Plant Pathology Research Institute, ARC, Giza, by using the susceptible maize cultivar Boushy cv.

Inoculum preparation of the pathogenic fungus *M. maydis*

Each *M. maydis* isolate was grown on potato dextrose agar (PDA) supplemented with 2 g/L yeast extract at 28°C in the dark for 6 days, until complete surface colonization was achieved. Subsequently, 1 cm^2 agar disk from the cultured plate was aseptically transferred to inoculate a 500-ml bottle containing 150 g of sorghum grain sterilized by autoclaving for 30 minutes. The inoculated bottles were incubated for two weeks at 26 ± 2°C until the sorghum grains were completely colonized and preserved until soil inoculation **(Zeller *et al.* 2002)**.

Soil infestation

Magnaporthiopsis maydis inoculum (5% w/w) was carefully mixed with autoclaved clay loam soil **(Samra *et al.* 1966)**. Before planting, the infected soil was transferred to sterilized pots (30 cm in diameter), watered, and allowed to adapt for a week. Maize grains (cv. Boushy) were surface sterilized for 2 minutes with a 2.5% sodium hypochlorite solution, then rinsed several times for 5 minutes with sterilized distilled water from each pot. Five grains were planted for each treatment, with four pots serving as duplicates.

Disease assessment

Disease incidence was defined by **Sabet *et al.* (1966)** as the percentage of infection 90 days post-sowing. Thirty-five days after silking**, El-Shafey *et al.* (1988)** recorded the levels of disease incidence in the surviving plants as follows:

$$\text{Disease incidence (DI \%)} = \frac{\text{No. of infected plants}}{\text{No. of total plants}} \times 100$$

The treatment efficiency in reducing late wilt disease incidence was evaluated using the formula proposed by **Rewal and Jhooty (1985)**

$$\textbf{Efficiency (\%)} = \frac{C - T}{C} \times 100$$

Where: C = infection (%) in the negative control,
T = infection (%) in the treatment.

Molecular identification

The identification of the most virulent pathogen isolate, El-Menia *M. maydis*-$_2$. The isolated genomic DNA was extracted in CTAB buffer and utilized for molecular identification. Polymerase chain reactions (PCR) were subsequently carried out using the universal fungal primers ITS1 and ITS4 to amplify the ribosomal DNA (rDNA) region **(White *et al.* 1990; Yousef 2021).** The sequencing procedure occurred at Macrogen Corp., Korea. The resultant DNA sequences have been submitted and archived in the NCBI GenBank for public accessibility and citation reasons.

Synthesis and characterization of chitosan nanoparticles

Nanoparticles of chitosan have been prepared in Mycology & Disease Survey Research "Dep. Nanotechnology & Advanced Nano-Materials Laboratory (NANML)" by

using the ionic gelation method. Chitosan was solubilized at a concentration of 0.5% (w/v) in 1% (v/v) acetic acid and then adjusted to a pH of 4.6-4.8 using 10 N sodium hydroxide. Chitosan nanoparticles were generated spontaneously by adding 1 ml of a 0.25% (w/v) aqueous tripolyphosphate solution to 3 ml of chitosan solution under continuous magnetic stirring. Centrifugation was used to purify the nanoparticles for 30 minutes at 9000 rpm. After removing the supernatants, the chitosan nanoparticles were thoroughly washed with distilled water to eliminate any residual sodium hydroxide, then freeze-dried prior to subsequent use or analysis (**Qi *et al*., 2004**).

Characterization

The study characterized chitosan nanoparticles using various measurement techniques. Zetasizer Nano-ZS90 was employed to evaluate the Zeta potential and particle size distribution. Fourier-transform infrared (FTIR) spectra were acquired using potassium bromide pellets. Atomic force microscopy was used to visualize nanoparticles on silicon substrates. A D/max-rA diffractometer was used to get X-ray powder diffraction patterns. Zetasizer analysis was also conducted to assess the average size and distribution of the nanoparticle suspension batches.

Evaluation of chitosan and chitosan nanoparticles against *M. maydis* and *T. harzianum in vitro*

Chitosan and chitosan nanoparticles at concentration of 50, 100, 150, and 200 ppm were evaluated against *M. maydis* and *T. harzianum in vitro* on potato dextrose agar (PDA) plates by adding the respective amounts to flasks containing PDA liquid medium to achieve the final concentrations. This mixture was then poured into 9 cm diameter Petri dishes, testing each concentration in three replicates. Additionally, three untreated PDA plates served as controls. A disc 5 mm obtained from a 5-day-old culture of either *M. maydis* or *T. harzianum* was inoculated in the middle of each Petri dish. The plates were incubated at 27°C and observed daily until the tested fungus of the control treatment completely covered the surface of the plate. The linear growth and percentage reduction were subsequently examined using the formula provided by **Yeh and Sinclair (1980)**.

$$G = C\text{-}T/C \times 100$$

Where:

G = Percentage of fungal growth reduction.

C = Fungal growth in the control (Pathogen alone)

T = Fungal growth in the treatment (Pathogen with the tested chemical).

Evaluation of the antagonistic *T. harzianum* isolate against *M. maydis* with various concentrations of chitosan and chitosan nanoparticles *in vitro*

Dual culture assays using PDA plates were conducted to evaluate the inhibitory effects of *T. harzianum* on *M. maydis* in the presence of varying concentrations of chitosan and chitosan NPs, according to **Dennis and Webster (1971)**. A 9 cm diameter plate filled with PDA medium mixed with chitosan and chitosan nanoparticles at various concentrations was inoculated on one side using a disc with a 5 mm diameter derived from the periphery of a 7-day-old culture of *M. maydis*. The opposite side of the plate was inoculated with a comparable disc of *T. harzianum* isolate derived from a 3-day-old culture. Three plates were utilized for each treatment. Control plates contained only the pathogenic fungus inoculated in the PDA medium, while other control (0 concentration), untreated PDA plates, were inoculated with *T. harzianum* against *M. maydis*. All inoculated plates were incubated at 27°C for 7 days, monitored daily until

M. maydis growth covered the plate as a control. Subsequently, mycelial growth and the percentage of growth reduction of the pathogenic fungus as mentioned before by **Yeh and Sinclair (1980)**.

Transmission Electron Microscopy (TEM)

To study the effect of chitosan and chitosan NPs on the vegetative mycelium of the pathogen, these steps were followed: fungal specimens, approximately 1 mm^3 each, were extracted from agar colonies for TEM preparation. The samples were post-fixed in potassium permanganate solution for five minutes after being fixed in 3% glutaraldehyde and washed in phosphate buffer at room temperature. For 15 minutes in each alcohol dilution, the samples were dehydrated in an ethanol series ranging from 10% to 90%, and then for 30 minutes in absolute ethanol. Through a graded sequence, samples were infiltrated with acetone and epoxy resin before being penetrated with pure resin. Copper grids were used to capture ultrathin sections. After that, sections were double-stained in uranyl acetate, then the lead sections that were stained were observed with a JEOL - JEM 1010 TEM at 70 kV at the Regional Centre for Mycology and Biotechnology (RCMB), Al-Azhar University **(Amin *et al.*, 2022 and 2024)**.

Evaluation of *T. harzianum*, chitosan, and chitosan nanoparticles against *M. maydis* late wilt disease under greenhouse conditions

This experiment was conducted at the Maize and Sugar Crops Disease Research Department, Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza. *T. harzianum*, both individually and in combination with chitosan, and chitosan nanoparticles (NPs) at three concentrations (50, 100, and 150 ppm) were evaluated against isolate El-Minia *M. maydis*-2 late wilt disease under greenhouse conditions. The preparation of inoculum for *M. maydis* and the soil infestation process were mentioned before in the pathogenicity test. *T. harzianum* was prepared by culturing 5mm mycelial discs from the 4-day-old culture of the fungus in 250 ml conical flasks containing potato dextrose broth medium (**El-Gammal *et al.*, 2025**). The cultures were kept at 25 ± 2 °C for 10 days of incubation to produce a spore suspension with a concentration of 6×10^6 spore/ml.

Five kernels of the maize (Boushy cv.) were cultivated in each pot (30 cm in diameter) after being soaked in each treatment, chitosan or nano-chitosan at concentrations of 50, 100, and 150 ppm for 6 hours. The kernels were air-dried, while the ones soaked in water served as a control. The experimental treatments were organized using a randomized complete block design (RCBD). For each treatment, four pots containing 5 plants and all prescribed cultural practices were rigorously implemented. Disease incidence was evaluated 90 days after planting, with the results recorded as outlined previously in the pathogenicity test.

Enzyme activity

The effect of treating maize grains with *T. harzianum*, only and in combined with chitosan, and chitosan NPs at (50, 100, and 150 ppm) concentration against *M. maydis*-$_2$ late wilt disease on enzyme activities (peroxidase, catalase, and β-1,3-glucanase), were determined in the maize plant leaves, taken 30 days after application under greenhouse conditions **(Maxwell and Bateman, 1967).**

Extraction of enzymes and bioassay

In a mortar, 0.1 M sodium phosphate buffer at pH 7.1 was used to homogenize approximately 2 g of

maize leaves from each treatment at a rate of 2 ml per gram of fresh-weight tissues. The homogenized tissues were passed through four layers of cheesecloth for filtration, and the resulting filtrate was centrifuged at 3000 rpm for 15 minutes at 6°C. The transparent supernatant was collected and considered as a crude enzyme extract for analysis. The supernatant was stored at -20°C in the refrigerator for preservation until the enzyme activity was assessed.

β-1, 3-glucanase assay:

β-1,3-glucanase enzyme activity was measured following the method outlined by **Abeles and Forrence (1970)**, 0.5 ml of 0.05 M potassium acetate buffer (pH 5) containing 4% Laminarin was combined with 0.5 ml of enzyme extract. Incubation of the mixture was carried out at 40 °C for one hour. The reaction is stopped by the addition of 1 ml of dinitrosalicylic acid, and then the mixture is heated in a boiling water bath at 100°C for five minutes, and the test tubes are allowed to cool. Subsequently, 3 mL of distilled water was added. The activity of β-1,3-glucanase was quantified as the variation in absorbance per minute at 500 nm. Enzyme activity was measured as micromoles of glucose equivalents produced per gram of fresh tissue per minute.

Peroxidase activity

Peroxidase activity was determined following the method of **Allam and Hollis (1972**) by measuring the oxidation of pyrogallol in the presence of hydrogen peroxide (H2O2), with absorbance recorded at 425 nm. A total reaction volume of 3.0 mL was prepared by combining 0.5 mL of enzyme extract, 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.0), 0.3 mL of pyrogallol, and 0.1 mL of 1.0% hydrogen peroxide, with the remaining volume made up using distilled water. The change in absorbance at 425 nm per minute per gram of fresh maize tissue was used as an indicator of peroxidase activity.

Catalase activity

The activity of catalase was assessed according to the method described by **Ueda *et al.* (1990)**. The reaction mixture was prepared by combining 0.2 ml of crude extract, 0.5 ml of 0.2 M sodium phosphate buffer at pH 7.6, and 0.3 ml of 0.5% hydrogen peroxide. This was followed by bringing the final volume up to 3 ml with distilled water. The enzyme activity was determined as the variation in absorbance per minute per gram of fresh maize tissue at 240 nm.

Evaluation of *T. harzianum*, chitosan, and chitosan nanoparticles against *M. maydis* late wilt disease under field conditions

The effect of *Trichoderma harzianum*, individually and in combination with chitosan, or chitosan nanoparticles (chitosan-NPs) at concentrations of 50, 100, and 150 ppm, as grains soaking, against late wilt disease incidence. Maize growth parameters and yield components were evaluated at Gemmieza and Malawy Agricultural Research Stations infected with a mixed inoculum of *M. maydis* during the 2024 growing season under field conditions.

In this respect, soaking the grains for 6 hours was done for each treatment, and the grains were also soaked with water used as the control. The experimental treatments were organized using a randomized complete block design (RCBD) with three replicates (plots). Each experimental plot comprises four rows measuring 80 cm in width and 6.0 m in length, with 20cm plant spacing, which were sown with 2 grains per hill and thinned to one plant per hill three weeks after planting. All agronomic methods recommended by the Ministry of Agriculture, Egypt, were implemented to grow maize plants;

fields were irrigated, fertilized, and pesticide spraying practices were excluded as usual.

Disease incidence was recorded after 90 days of planting, as mentioned before in the pathogenicity test. Growth parameters and yield characteristics, such as plant length (cm), stem diameter (cm), ear length (cm), ear weight (g), 100-kernel weight (g), and grain weight per plant (g), were documented ninety-nine days after planting.

Statistical Analysis

For both enzyme activities and *in vitro* experiments, the completely randomized design (CRD) was employed. Randomized complete block design (RCBD) was used for greenhouse and field environments. The data were analyzed using the statistical software ASSISTAT version 7.6 beta, created by **Silva and Azevedo (2009)**. Data were analyzed using ANOVA, and mean comparisons were performed using the least significant difference (LSD) test at a significant level of $P \leq 0.05$.

RESULTS

Pathogenicity test

Data in Table 1 indicate that there was a significant variation in the virulence of all tested *M. maydis* pathogenic isolates on Boushy cv. maize plants under greenhouse conditions. El-Minia isolate, *M. maydis*-$_2$, exhibited the highest virulence, resulting in a disease incidence of 76%. In contrast, the Sakha isolate, *M. maydis*-$_5$, demonstrated the lowest virulence, with a disease incidence of only 24%.

Molecular identification

The ITS-rDNA fragments from the highest virulent isolate (Isolate El-Minia *M. maydis*-$_2$) were amplified using ITS1 and ITS4 primers. Figure 1 indicates that the purified polymerase chain reaction (PCR) amplification was sequenced (*Magnaporthiopsis maydis*, (accession no. PV498746). A BLAST analysis of the GenBank database showed 100% identity with *M. maydis* (accession no KX306544.1).

Table 1. Pathogenicity test of six isolates of *M. maydis* on maize (Boushy cv.) *in vivo.*

Isolate No.	Source		
	Governorate	**Location**	**Disease incidence**
***M. maydis*-$_1$**	El-Minia	El-Minia	40
***M. maydis*-$_2$**		El-Minia	76
***M. maydis*-$_3$**	El-Beheira	El-Nubaria	36
***M. maydis*-$_4$**	El-Giza	El-Giza	32
***M. maydis*-$_5$**	Kafr-El-Sheikh	Sakha	24
***M. maydis*-$_6$**		Kleen	48
Control			0.0
L.S.D. at 0.05 %			19.76

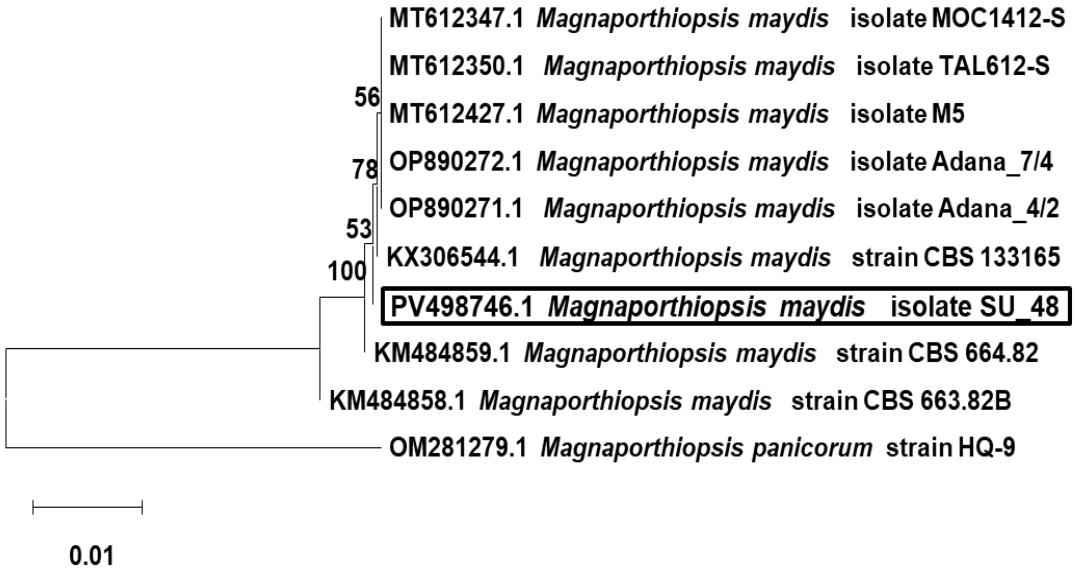

Fig. 1. Multiple sequence alignment of *M. maydis* GenBank (ITS: PV498746) and phylogenetic tree with the most likely *M. maydis* GenBank accession numbers.

Characterization of Chitosan Nanoparticles

Figure 2 depicts transmission electron microscopy images of chitosan nanoparticles ranging in size from 36 to 50 nm and having an irregular form (Fig 2).

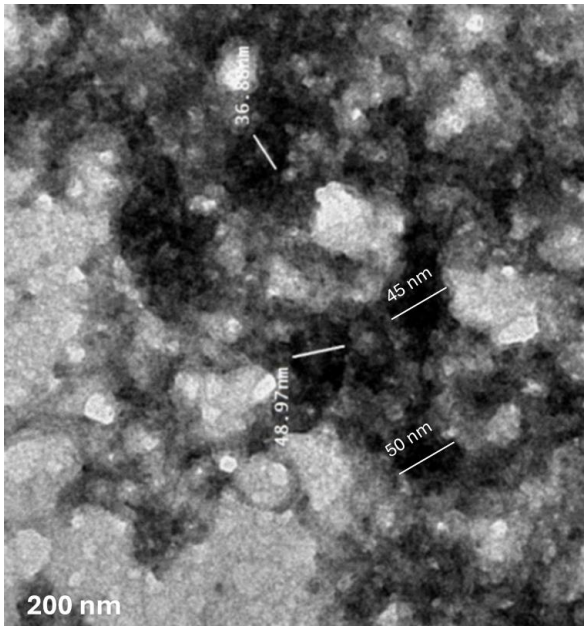

Fig. 2. Chitosan nanoparticles with size ranging from 36nm to 65nm were imaged using a transmission electron microscope.

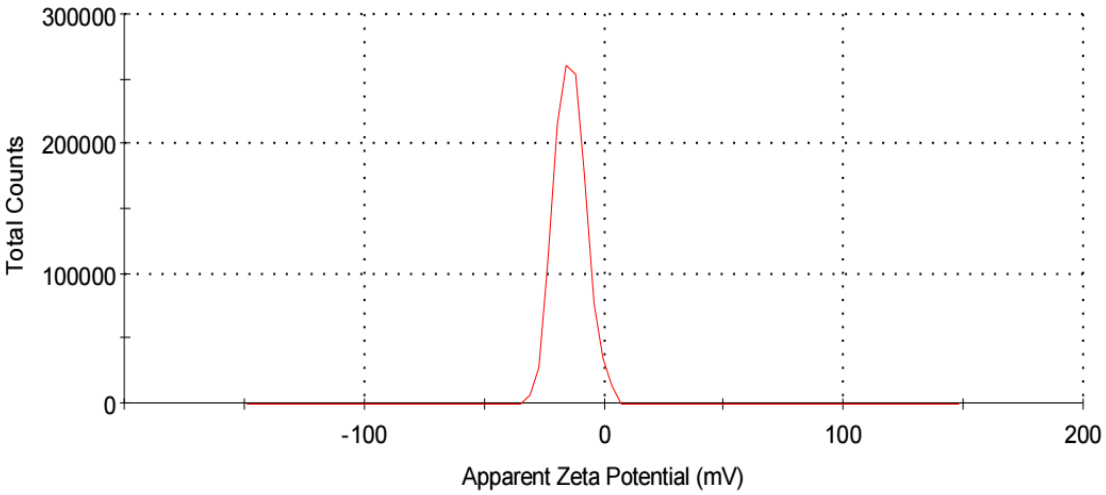

Fig. 3. Zeta potential of Chitosan nanoparticles.

A dynamic light scattering approach was used to determine the size distribution and stability of the produced chitosan nanoparticles (Fig 3). Zeta potential detected significantly negative charge particles, indicating strong electrostatic repulsion between particles, preventing them from aggregating and keeping the dispersion constant.

Data in Figure 4 shows two firm peaks in the X-ray diffraction patterns of chitosan nanoparticles at 10.4 and 21.8, indicating the low degree of crystallinity of chitosan nanoparticles. The XRD results suggested increased disorder in the chain alignment of the nanoparticles following crosslinking. The dynamic light scattering technique was employed to investigate the size distribution and stability of the prepared chitosan nanoparticles (Figure 3). Chitosan-NPs were produced with an average size of 15 ± 3 nm, as measured using a Nano ZS instrument from Malvern Instruments.

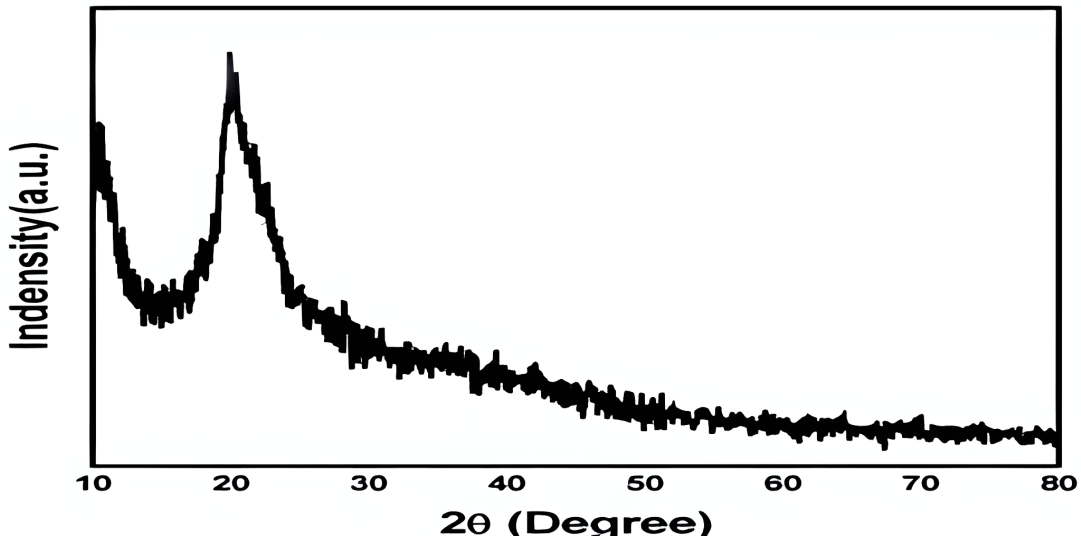

Fig. 4. X-ray diffraction (XRD) pattern of chitosan nanoparticles.

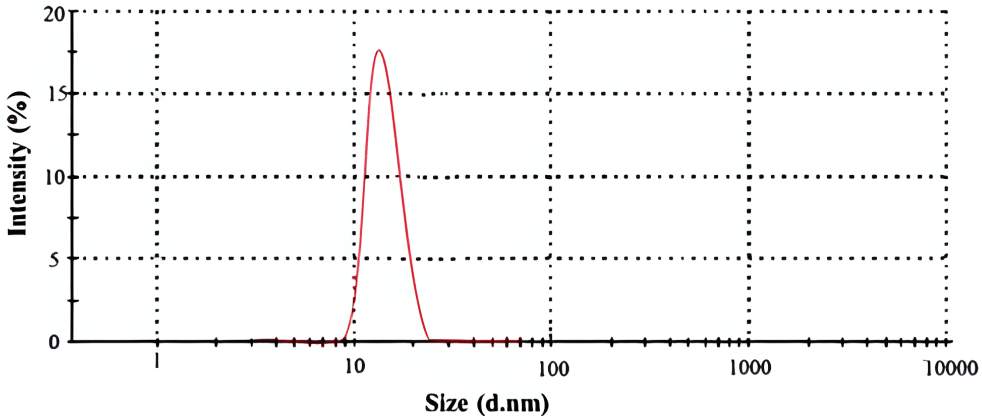

Fig. 5. Dynamic light scattering (DLS) of chitosan nanoparticles.

Evaluation of chitosan and chitosan nanoparticles against *M. maydis in vitro*

Data shown in Table 2 demonstrate that all treatments of chitosan and chitosan nanoparticles (NPs) at various concentrations significantly inhibit *M. maydis* linear growth compared with the control. In all tested concentrations, chitosan nanoparticles showed the highest efficacy in inhibiting the linear growth of *M. maydis*, reducing it to an average of 35.5 mm, achieving a growth reduction of 60.58%, followed by chitosan treatment, which resulted in an average linear growth of 48.4 mm and a growth reduction of 46.27%. Increasing the concentration of chitosan and chitosan NPs from 50 to 200 ppm gradually decreased the linear growth and increased the *M. maydis* growth reduction. The highest inhibitory effected on fungal growth was obtained at 200ppm of chitosan and chitosan NPs, which resulted in an average linear growth of 18.8 mm and a growth reduction of 79.11%. In contrast, at a concentration of 50 ppm, chitosan and chitosan nanoparticles showed the lowest average linear growth at 43.9 mm and a growth reduction of 51.22%.

Table 2. Evaluation of chitosan and chitosan nanoparticles effect on *M. maydis* linear growth and growth reduction on PDA plates *in vitro*

Concentration (ppm)	*M. maydis* linear growth (mm)			Reduction (%)		
	Chitosan	Chitosan (NPs)	Mean	Chitosan	Chitosan (NPs)	Mean
50	53.2	34.6	43.9	40.89	61.55	51.22
100	39.8	21.8	30.8	55.78	75.78	65.78
150	34.0	18.2	26.1	62.22	79.78	71.0
200	24.8	12.8	18.8	72.44	85.78	79.11
Control	90.0	90.0	90.0	0.00	0.00	0.0
Mean	48.4	35.5	-	46.27	60.58	-
L.S.D at 0.05	Concentration = C		1.07	Concentration = C		1.19
	Treatment = T		0.50	Treatment = T		0.53
	C x T		1.28	CxT		1.43

Evaluation of chitosan and chitosan nanoparticles (NPs) effect on *T. harzianum in vitro*

Data in Table 3 shows that all treatments of chitosan and chitosan nanoparticles at different concentrations decreased the linear growth of *T. harzianum* compared with the control; furthermore, *Trichoderma harzianum* mycelium growth remains

strongly unaffected by chitosan concentrations from 50 to 150 ppm, while it decreases significantly at concentrations 200 ppm. The highest growth of *T. harzianum* fungus was observed at a concentration of 50 ppm of chitosan and chitosan nanoparticles, with an average linear growth of 83.02 mm and a growth reduction of 7.76%. In contrast, at a concentration of 200 ppm, both chitosan and chitosan nanoparticles exhibited the least enhancement in fungal growth, with an average linear growth of 66.46 mm and a growth reduction of 26.16%.

Table 3. Evaluation of chitosan and chitosan nanoparticles on affecting the linear growth of *T. harzianum* isolate on PDA plates *in vitro.*

Concentration (ppm)	*T. harzianum* linear growth (mm)			Reduction (%)		
	Chitosan	Chitosan (NPs)	Mean	Chitosan	Chitosan (NPs)	Mean
50	83.7	82.34	83.02	7	8.51	7.76
100	78.26	76.42	77.34	13.04	15.09	14.07
150	75.64	72.5	74.07	15.96	19.44	17.7
200	70.28	62.64	66.46	21.91	30.4	26.16
Control	90	90	90	0	0	0
Mean	79.58	76.78	78.18	11.58	14.69	-
L.S.D at 0.05	Concentration = C 4.29 Treatment =T 1.92 CxT 5.18			Concentration = C 4.77 Treatment =T 2.13 CxT 5.76		

Evaluation of the antagonistic *T. harzianum* isolate against *M. maydis* with various concentrations of chitosan and chitosan nanoparticles *in vitro*

The data presented in Table 4 show that the antagonistic *T. harzianum* isolate demonstrated varying inhibitory effects against the *M. maydis* fungal pathogen when tested on PDA plates with different concentrations of chitosan and chitosan nanoparticles *in vitro*. This resulted in a significant reduction in fungal growth, ranging from 74.11 % to 86.0%, due to the increased concentration of chitosan and chitosan nanoparticles from 0 to 200 ppm compared to the control treatment.

Table 4. Evaluation of the antagonistic *T. harzianum* against *M. maydis* linear growth and growth reduction on PDA plates with different concentrations of chitosan and chitosan nanoparticles *in vitro.*

Concentration (ppm)	*M. maydis* linear growth (mm)			Reduction (%)		
	Chitosan	Chitosan (NPs)	Mean	Chitosan	Chitosan (NPs)	Mean
***0**	23.3	23.3	23.3	74.11	74.11	74.11
50	24.0	21.2	22.6	73.33	76.45	74.88
100	18.8	16.4	17.6	79.11	81.78	80.44
150	16.8	13.4	15.1	81.33	85.11	83.22
200	14.2	11.0	12.6	84.22	87.78	86.0
Control	90.0	90.0	90.0	0.00	0.00	0.0
Mean	31.18	29.22	-	65.35	67.54	-
L.S.D at 0.05	Concentration = C 1.06 Treatment = T 0.48 C x T 1.30			Concentration = C 1.19 Treatment = T 0.53 CxT 1.43		

***0 =** Untreated PDA plates were inoculated with *T. harzianum* against *M. maydis* only.

The highest inhibitory effect on the growth of the pathogenic fungus was observed at a concentration of 200 ppm of both chitosan and chitosan nanoparticles with *T. harzianum.* The average linear growth measured 12.6 mm at this concentration, and the growth reduction reached 86.0%. Meanwhile, chitosan and chitosan nanoparticles at 0 and 50 ppm resulted in average linear growth of 23.3 and 22.6 mm for *M. maydis*, with corresponding growth reduction of 74.11% and 74.88%, respectively.

Transmission Electron Microscopy (TEM)

TEM observations for the mycelium grown on PDA amended with treatment with chitosan and chitosan nanoparticles (NPs) led to clear cellular alterations in the mycelium of *M. maydis*. Ultra-thin sections of *M. maydis* mycelium treated with chitosan revealed partial lysis of the cell wall (CW), and shrinkage of the cell membrane (CM), leaving a large space between the cell wall; Cytoplasmic materials were concentrated at the center of the cell, with chitosan accumulating on the cell wall (indicated by arrows) (Fig.$_{6\text{-}B}$). While, mycelium treated with chitosan NPs (Fig.$_{6\text{-}C}$) reveled that both of cell wall and cell membrane structures were severely damaged lead to the death of the cell; the accumulation of chitosan NPs observed on the mycelium surface (arrows) compared with the non-treated (control) mycelium (Fig.$_{6\text{-}A}$); showed well-defined organelles; cell wall (CW), cell membrane (CM), nucleus (N), nucleolus (Nu), mitochondria (M) and septum (S) between hyphal cells.

Results obtained by TEM (Fig.7) show a great variation between a non-treated sample (control) of *T. harzianum* and treated ones. Treated mycelium with chitosan (Fig.$_{7\text{-}E}$) detected a transparent and irregular cell wall, the cytoplasmic contents represented slight changes with the appearance of chitosan on the hyphal surface (arrows) and numerous lipid granules (L). Amazingly, treated mycelium with chitosan NPs showed disintegration of the cell wall and cell membrane rupture, resulting in the loss of cellular content with accumulation of chitosan NPs (arrows) all over the *T. harzianum* mycelium (Fig.$_{7\text{-}F}$), compared with the non-treated (control) mycelium (Fig.$_{7\text{-}D}$) that displayed clearly defined organelles; nucleus (N) with its nucleolus (Nu), mitochondria (M), and an even cell wall (CW) and membrane (CM).

TEM observations on the effect of *T. harzianum* against *M. maydis* using chitosan and chitosan NPs are shown in Fig.$_{8.}$ Degeneration of *M. maydis* cell wall (Mm CW) and cell membrane (Mm CM) with cytoplasmic materials was observed, resulting from the effect of *T. harzianum* growth (Fig.$_{8\text{-}G}$) that had an intact cell wall (T CW) and cell membrane (T CM) with organized organelles, including cellular organelles, nucleus (N), and nucleolus (Nu). However, the effect of *T. harzianum* growth with the addition of chitosan (Fig.$_{8\text{-}H}$) showed a great effect against the pathogenic *M. maydis* hyphae with chitosan absorbance (arrows). In addition, the synergistic effect of *T. harzianum* growth with chitosan NPs represented complete inhibition of *M. maydis* with accumulation of chitosan NPs (arrows) (Fig.$_{8\text{-}I}$).

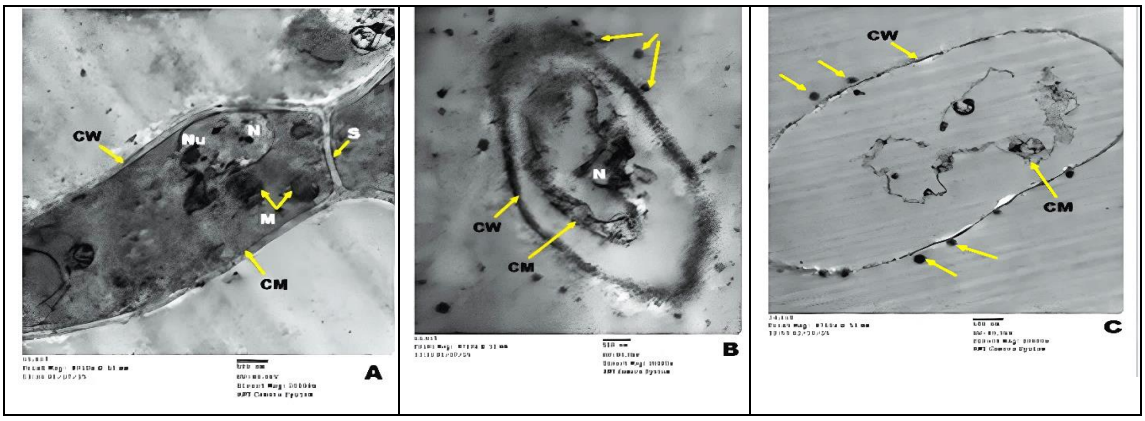

Fig. 6. Transmission electron micrographs *of M. maydis:* A, control; B, treated with chitosan bulk; and C, treated with chitosan NPs. (CW = cell wall; CM =cell membrane; M = mitochondrion; N = nucleus; Nu = nucleolus; S = septum. Magnification = 20000x.

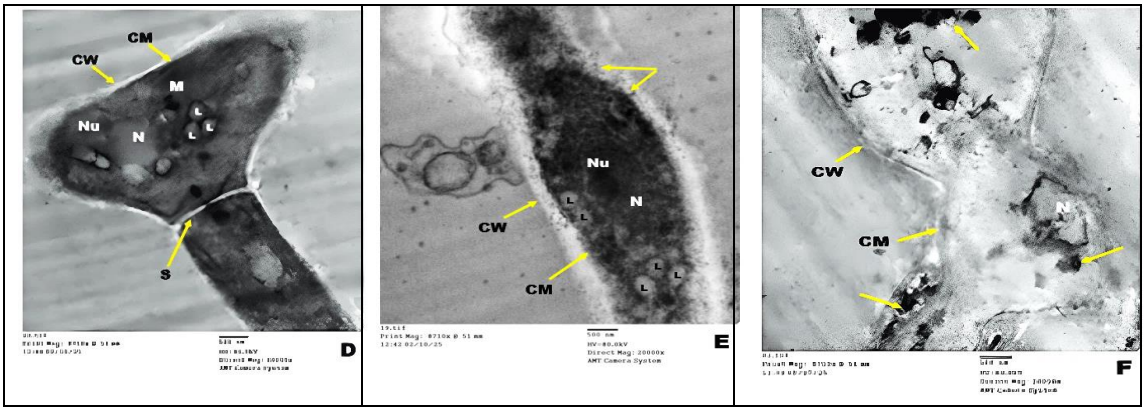

Fig.7. Transmission electron micrographs of *T. harzianum:* D; control, E; treated with bulk, and F; treated with chitosan NPs. (CW = cell wall; CM =cell membrane; M = mitochondrion; N = nucleus; Nu = nucleolus; L = lipid granules; S = septum. Magnification = 20000x.

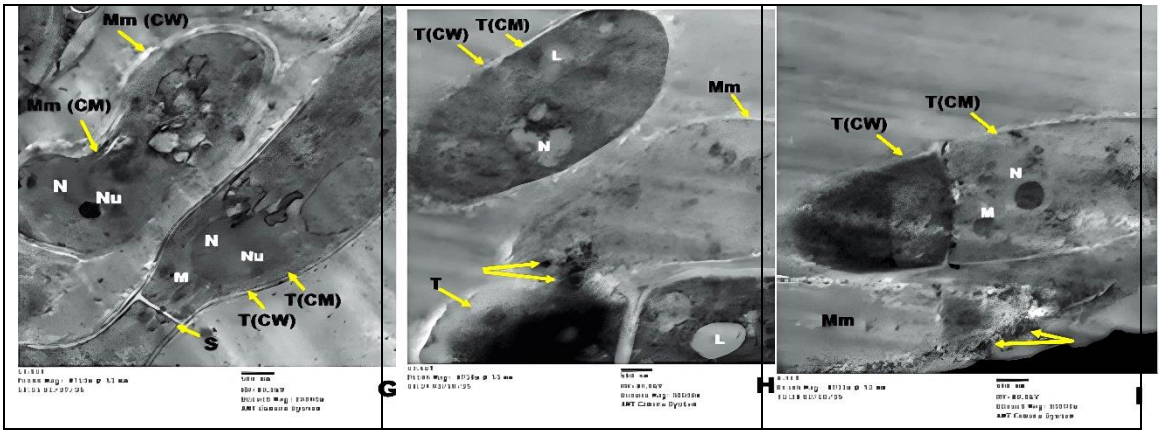

Fig.8. Transmission electron micrographs showed (G) *T. harzianum* and *M. maydis*, (H) *T. harzianum* and *M. maydis* treated with chitosan bulk, and (I) *T. harzianum* and *M. maydis* treated with chitosan NPs. (Mm = *M. maydis*; CW = cell wall; CM =cell membrane; M = mitochondrion; N = nucleus; Nu = nucleolus; L = lipid granules; S = septum. Magnification = 20000x.

Evaluation of *T. harzianum*, chitosan, and chitosan nanoparticles against *M. maydis* late wilt disease under greenhouse conditions

In Table 5, data evaluate the efficiency of *T. harzianum*, both individually and in combination with chitosan, and the impact of chitosan nanoparticles (NPs) at concentrations of 50, 100, and 150 ppm on *M. maydis* late wilt disease was assessed under greenhouse conditions. Compared to the infested control, late wilt disease was significantly decreased in all treatments. The combination of *T. harzianum* and chitosan nanoparticles at 150 ppm exhibited the greatest effectiveness in resistance to late wilt disease, achieving a 0.0% disease incidence and 100% efficiency. Following this, the combination of *T. harzianum* with chitosan (NPs) at 100 ppm, as well as *T. harzianum* with chitosan at 150 ppm, and chitosan (NPs) at 150 ppm alone, recorded disease incidences of 6.67% and efficiencies of 86.66%, respectively. Furthermore, the treatment with chitosan at 50 ppm showed the lowest effectiveness against the incidence of late wilt disease, with recorded incidences of 30.0% and an efficiency of 40.0%.

Table 5. Evaluation of *T. harzianum*, chitosan, and chitosan nanoparticles (NPs) individually or in combination against *M. maydis* late wilt disease *in vivo* under greenhouse conditions.

Treatments	Conc. (ppm)	Disease incidence (%)	Efficiency (%)
Chitosan	**50**	30.0	40.0
	100	26.67	46.66
	150	10.0	80.0
Chitosan (NPs)	**50**	20.0	60.0
	100	16.67	66.66
	150	6.67	86.66
***T. harzianum* +**	**50**	16.67	66.66
Chitosan	**100**	10.0	80.0
	150	6.67	86.66
***T. harzianum* +**	**50**	13.33	73.34
Chitosan (NPs)	**100**	6.67	86.66
	150	0.0	100.0
T. harzianum	**-**	16.67	66.66
Un-infested control	**-**	0.0f	100.0
Infested control	**-**	50.0	0
L.S.D at 0.05		11.99	-

Effect of *T. harzianum*, chitosan, and chitosan nanoparticles against *M. maydis* on enzyme activities under greenhouse conditions

Table 6 shows that all treatments of *T. harzianum*, alone or combined with chitosan and chitosan nanoparticles (NPs) at various concentrations, significantly enhanced the enzyme activities in leaves of Mize plants compared to infested and uninfested control.

The activity of β-1,3-glucanase enzyme was highly increased with the combination of *T harzianum* and 150 ppm chitosan (NPs), yielding an enzyme activity value of 4.146. This was followed closely by another combination of *T. harzianum* and 150 ppm chitosan, which recorded an

enzyme activity of 3.945. Conversely, the lowest increase in β-1,3-glucanase enzyme activity was noted with 50 ppm chitosan administered alone, resulting in an enzyme activity value of 1.862.

The combination of *T. harzianum* and 150 ppm chitosan (NPs) showed the highest increase in peroxidase activity, registering a value of 2.731. The combination of *T. harzianum* and 150 ppm chitosan came next, with a value of 1.950. However, using 50 ppm chitosan alone resulted in the lowest increase in peroxidase activity, with a value of 1.477.

The most significant increase in catalase activity was observed when the combination of *T. harzianum* and 100 ppm chitosan (NPs) was used, with a value of 2.042 enzyme activity. In contrast, the lowest catalase activity was recorded with the individual treatment of 50 ppm chitosan, which had an activity value of 1.23.

Table 6. Effect of *T. harzianum*, chitosan, and chitosan nanoparticles on *M. maydis* and their influence on enzyme activities under greenhouse conditions.

Treatments	Con. (ppm)	*β-1,3 gluconase activity	**Peroxidase activity	**Catalase activity
Chitosan	**50**	1.862	1.477	1.232
	100	2.001	1.724	1.589
	150	2.198	2.201	1.739
Chitosan (NPs)	**50**	1.947	1.940	1.306
	100	2.316	2.086	1.864
	150	2.467	2.254	1.750
***T. harzianum* +**	**50**	2.192	1.672	1.596
Chitosan	**100**	2.877	2.250	1.875
	150	3.945	2.619	1.950
***T. harzianum* +**	**50**	2.274	2.126	1.735
Chitosan (NPs)	**100**	3.464	2.366	2.042
	150	4.146	2.731	1.990
T. harzianum	**-**	2.442	2.192	1.425
Un-infested control	**-**	1.814	1.394	1.077
Infested control	**-**	1.172	1.286	0.873
L.S.D at 0.05		0.2181	0.1236	0.1113

*β-1,3-glucanase activity (µ mole Glucose/min /ml)
**Catalase & Peroxidase (absorbance/min/g fresh weight of maize)

Evaluation of *T. harzianum*, chitosan, and chitosan nanoparticles against *M. maydis* late wilt disease under field conditions

The effect of *T. harzianum*, individually and in combination with chitosan, and chitosan nanoparticles (NPs) at 50, 100, and 150 ppm concentrations, against late wilt disease incidence, maize growth parameters, and yield components was evaluated at Gemmieza and Malawy research stations, during the 2024 growing season under field conditions.

When compared to the control, data in Table 7 demonstrated a significant decrease in the incidence of late wilt disease across all tested treatments. Remarkably, the combination of *T. harzianum* with 150 ppm chitosan NPs reduced late wilt disease with the best efficiency, achieving 88.27%. This was closely followed by *T. harzianum* + 150 ppm chitosan and *T. harzianum* + 100 ppm chitosan NPs, which demonstrated efficiencies of 85.62% and 85.29%,

respectively, at Gemmieza station.

In Malawy station, the combination of *T. harzianum* 150 ppm + chitosan NPs exhibited the highest efficiency against late wilt disease, achieving an efficiency of 94.60%. Followed by the combination of chitosan NPs (100 ppm) + *T. harzianum* and 150 ppm chitosan NPs, which recorded efficiencies of 92.84% and 90.71%, respectively. In contrast, chitosan at 50 ppm exhibited the lowest effectiveness in reducing the incidence of late wilt disease, with efficiencies of 68.38% and 73.99% at the Gemmieza and Malawy stations, respectively.

Table 7. Evaluation of *Trichoderma harzianum*, chitosan, and chitosan nanoparticles (NPs) individually or in combination against the incidence of *M. maydis* disease at Gemmieza and Malawy stations during the 2024 growing season under field conditions

Treatments	Con. (ppm)	Gemmieza station		Malawy station	
		DI (%)	Efficacy * %	DI (%)	Efficacy * %
Chitosan	**50**	13.26	68.38	10.02	73.99
	100	11.59	72.37	7.26	81.16
	150	9.79	76.66	4.23	89.02
Chitosan (NPs)	**50**	11.73	72.03	8.35	78.33
	100	10.14	75.82	6.27	83.73
	150	8.85	78.90	3.58	90.71
***T. harzianum* + Chitosan**	**50**	9.06	78.40	6.36	83.49
	100	7.82	81.35	4.49	88.35
	150	6.03	85.62	3.97	89.70
***T. harzianum* + Chitosan (NPs)**	**50**	8.16	80.54	4.63	87.98
	100	6.17	85.29	2.76	92.84
	150	4.92	88.27	2.08	94.60
T. harzianum	**-**	7.02	83.26	5.32	86.19
Infested control	**-**	41.94	-	38.53	-
L.S.D at 0.05		1.83	-	1.42	-

In tables 8 and 9, all tested treatments significantly increased maize growth parameters and yield characteristics, including, plant length (cm), stem diameter (cm), ear length (cm), ear weight (g), 100-kernel weight (g), and grains weight per ear (g) when compared to the control at both Gemmieza and Malawy stations. The combination of chitosan nanoparticles (NPs) at 150 ppm and *T. harzianum* proved to be the most successful treatment for maize growth measures and yield components. This was followed by the combination of *T. harzianum* with 150 ppm chitosan, and *T. harzianum* with 100 ppm chitosan (NPs) alternately. In contrast, the treatment with chitosan at 50 ppm was found to be the least effective.

Table 8. Evaluation of *Trichoderma harzianum*, chitosan, and chitosan nanoparticles (NPs) on yield and growth parameters of maize at Gemmieza station during the 2024 growing season under field conditions.

Treatments	Conc.	Plant length (cm)	Stem diameter (cm)	Ear length (cm)	Ear weight (g)	100-kernels weight (g)	Grain weight/ear (g)
Chitosan	**50**	234.77	4.87	16.32	264.50	30.43	189.91
	100	239.13	5.17	16.99	276.50	32.20	205.67
	150	246.33	5.53	17.30	290.67	33.57	218.11
Chitosan (NPs)	**50**	241.23	5.23	16.80	324.53	32.23	214.33
	100	245.17	5.73	18.47	330.53	35.60	226.48
	150	248.13	6.70	18.67	354.47	36.13	230.26
***T. harzianum* +**	**50**	241.67	5.57	16.78	322.73	35.65	220.41
Chitosan	**100**	247.57	6.13	17.83	339.27	37.90	232.39
	150	249.77	6.73	18.47	358.80	40.67	246.03
***T. harzianum* +**	**50**	243.73	5.83	18.10	348.33	38.43	239.73
Chitosan (NPs)	**100**	248.43	6.67	19.23	356.37	42.37	254.42
	150	252.67	6.93	21.21	380.83	45.80	271.32
T. harzianum	**-**	244.93	5.67	17.63	294.53	31.17	221.99
Infested control	**-**	217.43	3.57	12.77	192.37	24.77	149.21
L.S.D at 0.05		3.85	0.96	1.55	16.78	2.52	13.75

Table 9. Evaluation of *Trichoderma harzianum*, chitosan, and chitosan nanoparticles (NPs) on yield and growth parameters of maize at Malawy station during the 2024 growing season under field conditions.

Treatments	Conc.	Plant length (cm)	Stem diameter (cm)	Ear length (cm)	Ear weight (g)	100-kernels weight (g)	Grain weight/ear (g)
Chitosan	**50**	238.27	4.40	16.57	282.52	33.07	197.17
	100	244.03	4.67	17.91	295.83	34.87	212.83
	150	250.67	5.33	18.44	313.45	36.91	225.20
Chitosan (NPs)	**50**	239.27	4.80	17.83	330.18	35.53	223.93
	100	246.37	5.10	18.93	343.60	37.6	229.25
	150	252.47	5.73	19.60	368.79	39.16	238.97
***T. harzianum* +**	**50**	245.37	4.77	17.93	342.03	38.4	228.78
Chitosan	**100**	249.80	5.23	20.10	363.07	41.37	240.72
	150	255.20	6.27	20.33	373.75	43.07	251.81
***T. harzianum* +**	**50**	246.47	5.27	19.22	354.37	42.9	242.65
Chitosan (NPs)	**100**	253.97	5.97	20.53	369.47	47.57	248.33
	150	257.63	6.33	22.57	383.27	50.39	284.63
T. harzianum	**-**	248.53	5.17	18.40	327.69	39.8	231.33
Infested control	**-**	221.33	3.83	13.50	214.70	28.28	157.56
L.S.D at 0.05		5.01	0.72	1.11	19.82	2.51	15.34

DISCUSSION

One important fungal disease that impacts maize in Egypt is late wilt. During the flowering stage and right before maturity, it causes maize plants to wilt rapidly and quickly (**Sabet *et al.*, 1966; Payak *et al.*, 1970; El-Naggar *et al.*, 2015**). This disease can lead to substantial yield losses of up to 40%, with infection rates reaching 80-100% in heavily infested fields where

sensitive maize varieties are cultivated **(El-Hosary *et al*., 2015)**.

In this research, all the tested pathogenic isolates of *Magnaporthiopsis maydis* demonstrated significant variation in their virulence on Boushy cv. maize plants *in vivo*. El-Minia-$_2$ isolate exhibited the highest virulence, whereas the Sakha isolate displayed the lowest virulence. This conclusion coincided with the findings of **Zeller *et al.* (2000)** and **Hassan *et al.* (2022)**, who discovered significant variability in virulence among various *Harpophora. maydis* isolates through isolation trials on late wilt disease symptoms in Egyptian maize plants.

Chitosan nanoparticles (NPs) were created with a 15 ± 3 nm average size, as measured using a Nano ZS instrument. Research indicates that nanoparticles possess exceptional physical and chemical features as a result of their size., distribution, and shape compared to their bulk counterparts **(Elizabeth *et al*., 2019; El-Saadony *et al*., 2021)**. The effectiveness of chitosan is significantly enhanced when it is used in nanoparticle form, which typically ranges from 10 to 80 nm in size. This makes chitosan-based nanomaterials highly attractive as carriers (**Osuwa & Anusionwu, 2011; Chandra *et al*., 2015**).

This study highlights the application of safe management in controlling late wilt disease. *T. harzianum* as a biocontrol agent, and chitosan or chitosan nanoparticles (50, 100, 150, and 200 ppm), alone or in combinations, were used in controlling late wilt disease *in vitro* and *in vivo*. *Magnaporthiopsis maydis's* radial growth was suppressed by all examined treatments *in vitro* at varying treatments, which resulted in a significant decrease in its presence on PDA as compared to the control treatment. An increase in the concentration of chitosan and chitosan NPs from 50 to 200 ppm gradually increased the *M. maydis* growth reduction. The results were consistent with those mentioned by **Hassan *et al.* (2022),** who verified that, as compared to alternative treatments, *in vitro* chitosan nanoparticles demonstrated the strongest antifungal action at concentrations of 5 mM and 10 mM, inhibiting the phytopathogen *Harpophora maydis* (*M. maydis*) from growing radially.

In contrast to the control, chitosan and chitosan nanoparticles affect *T. harzianum* growth *in vitro*; furthermore, *Trichoderma harzianum* mycelium growth remains strongly unaffected by chitosan concentrations from 50 to 150 ppm. However, when the concentration reaches 200 ppm, the growth begins to significantly decrease. This is consistent with the results of **Zavala-González *et al.* (2016) and Kappel *et al.* (2022)** found that different strains of *Trichoderma* exhibit varying levels of tolerance to chitosan when grown on solid media, depending on the concentration of chitosan present. Specifically, *T. atroviride* and *T. koningiopsis* demonstrate tolerance to chitosan, even at a high concentration of 2 mg/ml on PDA medium. This tolerance may provide *Trichoderma* with a competitive advantage over fungal pathogens like *Fusarium oxysporum*. In contrast, we did not observe any adaptation to chitosan in sensitive plant pathogens, even after extended incubation periods. Chitosan and chitosan nanoparticles have unique antimicrobial properties (**Xing *et al*., 2015; El Gamal *et al*., 2016)**. Their biocompatibility and low toxicity (**Mahmood *et al*., 2017**); furthermore, chitosan nanoparticles are effective as attractive carriers for various applications (**Osuwa & Anusionwu, 2011; Chandra *et al*., 2015**).

The antagonistic isolate of *T. harzianum* showed varying levels of inhibition against the fungal pathogen *M. maydis* when tested on PDA plates with different concentrations of chitosan and chitosan nanoparticles *in vitro*. The inhibitory effects of chitosan and chitosan nanoparticles increased with concentration, rising from 0 to 200 ppm compared to the control. The outcomes align with the conclusions of **Kubicek *et al.* (2019)** and **Di Marco *et al*. (2022)**, who noted that various *Trichoderma* spp. specifically, *T. asperellum, T. harzianum, T. hamatum*, and *T. viride* are frequently employed as biological control agents to manage several pathogenic fungi due to their mycoparasitic properties. In a combined activity involving chitosan and *T. atroviride*, a strong synergistic effect was observed on the growth inhibition of significant plant pathogens, including *F. oxysporum*, and *C. beticola*, resulting in a 93% increase in inhibition (**Kappel *et al*., 2020**).

TEM observations showed cellular alterations in *M. maydis* mycelium due to treatment with chitosan and chitosan NPs, including partial lysis of the cell wall and shrinkage of the membrane, leaving a large space between cell walls. Chitosan NPs showed that both the cell wall and the cell membrane structures were severely damaged, which caused the cell death; the accumulation of chitosan NPs was observed on the mycelium surface, in contrast to the untreated control, which showed well-defined organelles. The combined effect of *T. harzianum* growth with chitosan and chitosan NPs demonstrated significant efficacy against the pathogenic *M. maydis* hyphae, leading to complete inhibition of *M. maydis* and the accumulation of chitosan NPs. Furthermore, according to some research, nanoparticles might alter the structure of microbial cell membranes, which would lead to cytoplasmic leakage and ultimately cell death **(Sawai and Yoshikawa, 2004; Brayner *et al*., 2006; Shoala *et al*., 2021)** and increases in the hyphal cell walls' thickness and cytoplasmic membrane integrity **(Li *et al,* 2009; Ansari *et al*., 2021; Mustafa *et al*., 2021).** The application of salicylic acid NPs, along with their natural forms, significantly reduced the thickness of the cell wall and induced cytoplasmic shrinkage in *Alternaria alternata* (**Abdel-Rahman *et al*., 2021; Hashem *et al*., 2022)**. Additionally, nanoparticles enhance antibiotic reaction rates on various bacteria in a synergistic manner (**El-Deeb *et al*., 2018)**. Selenium nanoparticles enhance antibiotic antimicrobial activity by bonding with antibiotics, resulting in DNA unraveling and cell wall breakdown, leading to cell death, as suggested by **Krishna *et al*. (2015)** and **Makhlof *et al*. (2023).**

The current study's *in vivo* findings confirmed the *in vitro* findings that all treatments, whether in the field or the greenhouse, significantly decreased the infection of late wilting disease. Additionally, *in vivo* results indicate that *T. harzianum*, both individually and in combination with chitosan, and chitosan (NPs) efficiently at three concentrations (50, 100, and 150 ppm) reduced disease incidence and enhanced plant resistance to the late wilt disease caused by *M. maydis*, compared to other treatments. Among these, the combination of *T. harzianum* + chitosan NPs at 150 ppm showed the highest efficiency, followed by the combination of *T. harzianum* with chitosan (NPs) at 100 ppm. As reported by **Al-Hetar *et al.* (2011), Sunpapao and Pornsuriya (2014), Silva *et al.* (2014), and Li *et al*. (2018),** this supports the findings of numerous studies about the potential of chitosan and *Trichoderma* spp. to control disease. The synergistic effect of *T.*

atroviride and chitosan on the inhibition of phytopathogens occurs through different mechanisms. Chitosan directly inhibits the pathogens through a biocidal mechanism, while the growth inhibition caused by *T. atroviride* is likely due to the secretion of secondary metabolites and enzymes that facilitate parasitism and ultimately kill the host (**Kappel *et al*., 2020**). *T. harzianum* can influence late wilt disease, as evidenced by studies conducted by **Abd El-Motty *et al*. (2010)**, **Li *et al*. (2018),** and **El-Shahawy and El-Sayed (2018)**. This effect is attributed to its production of secondary metabolites that exhibit antibiotic properties, which make it an effective biocontrol agent against plant pathogenic fungi (**Kubicek *et al*., 2019**). Chitosan and nano-chitosan have unique antimicrobial properties (**El Gamal *et al*., 2016**), which can significantly influence physiological processes, secondary metabolite synthesis, active chemical production, and plant development (**Jiang *et al*., 2007; Hassan *et al*., 2022**). Its biocompatibility and low toxicity make it an effective preventive mechanism for plants against pathogen attack (**Mahmood *et al*., 2017)**.

In this study, grains of maize treated with *T. harzianum*, alone or combined with chitosan and chitosan nanoparticles at various concentrations in the presence of the pathogenic fungus, significantly enhanced the enzyme activities in leaves of maize compared to infested and uninfested control. *T. harzianum* + 150 ppm chitosan (NPs) produced the greatest increases in β-1,3-glucanase, peroxidase, and catalase in the treated plant, followed by *T. harzianum* + 150 ppm chitosan, and *T. harzianum* and 100 ppm chitosan (NPs) treatments. In contrast, the treatment with 50 ppm chitosan was found to be the least effective *Trichoderma species* are capable of producing bioactive compounds that can be employed as biocontrol agents against plant pathogenic fungi, such as secondary metabolites like chitinase and chitinolytic enzymes and cell wall-degrading enzymes. (**Druzhinina *et al*., 2018; Kubicek *et al*., 2019)**. Meanwhile, extracellular enzymes and metabolites may be released as a result of nanoparticles' inhibitory effects. (**Alejandro and Rubiales 2009)**. The plants' antioxidant defense system, which includes the synthesis of antioxidant enzymes, including catalase (CAT), peroxidase (POX), and polyphenol oxidase (PPO), helps them deal with the buildup of reactive oxygen species (ROS) (**Khalifa *et al*., 2016; Alafari *et al*., 2024)**.

The effects of *T. harzianum* both alone or in combination with chitosan, and chitosan (NPs) effectively control late wilt disease incidence, enhance maize growth parameters, and yield components, in contrast to the control at Gemmieza and Malawy stations, during the 2024 growing season in the field. Notably, *T. harzianum* plus 150 ppm of chitosan nanoparticles achieved the highest efficiency in reducing late wilt disease, and elevated maize yield characteristics and growth parameters at both Gemmieza and Malawy research stations. This confirms what many researchers have found: The combination of chitosan and *Trichoderma atroviride* exhibits a synergistic effect against various plant pathogens. This effectiveness is attributed to the increased production of secreted hydrolytic enzymes by *T. atroviride*, along with other potential effectors, enhancing its ability to combat phytopathogens (**Kappel *et al*., 2020**). *Trichoderma species'* antagonistic characteristics against plant-pathogenic fungi can significantly reduce plant diseases, enhance crop tolerance, and promote plant growth (**Druzhinina *et al*., 2018;**

Kubicek *et al*., 2019). They are in competition with these plant pathogens for resources, reducing their prevalence by creating antibiotics, parasitism, or making host plants more resistant (**Berg *et al*., 2007**). *T. atroviride* is also capable of producing indole-3-acetic acid (IAA) and volatile organic compounds, such as 6-pentyl-2H-pyran-2-one (6-PP), both of which are known to promote plant growth (**Reithner *et al*., 2005**). Additionally, chitosan can induce the host plant's defense mechanisms and promote plant growth and protection because of its superior bioactivity, non-toxicity, and biodegradability. (**Xing *et al*., 2015; Mahmood *et al*., 2017; Li *et al*., 2018; Hassan *et al*., 2022).** Chitosan acts as an elicitor for plant defense mechanisms by increasing the levels of phenylalanine ammonia-lyase (PAL), an enzyme involved in the synthesis of phytoalexins, as well as various genes related to oxidative stress. These compounds have been demonstrated to enhance pathogen resistance and support overall plant growth (**Miya *et al*., 2007; Badawy and Rabea, 2011**).

CONCLUSIONS

The application of *T. harzianum*, individually and in combination with chitosan and chitosan NPs, at various concentrations in the greenhouse or in the field, attained the maximum level of efficiency in increasing enzyme activities, decreasing the late wilt disease, and improving maize growth parameters and yield components spatially Particularly, the combination of *T. harzianum* and chitosan NPs at a concentration of 150 ppm was particularly effective.

List of Abbreviations

RCBD	complete block design
PDA	Potato dextrose agar
g	Gram
cm	Centimeter
mm	Millimeter
LWD	Late wilt disease
ml	Milliliter
rpm	Revolutions Per Minute
nm	Nanometer equal to one billionth of a meter
NPs	Nanoparticles
ppm	Parts Per Million
CTAB	Cetyltrimethylammonium bromide
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
rDNA	Recombinant DNA
bp	base pair
ITS	Internal transcribed spacer
TE	Tris-EDTA
NCBI	National Center for Biotechnology Information
TEM	Transmission Electron Microscopy
kV	Kilovolt (a unit of electric potential)
w/v	weight per volume
v/v	volume per volume
min	Minute
pH	Potential of hydrogen
cv.	Cultivar
In Vitro	In Laboratory
In Vivo	Under Greenhouse and Field conditions
Fig.	Figure
AVOVA	Analysis of Variance

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الملخص العربي :

تحسين كفاءة فطر تريكوديرما هارزيانوم (*Trichoderma harzianum*) في مكافحة مرض الذبول المتأخر في الذرة باستخدام الكيتوزان وجسيمات الكيتوزان النانوية

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يُعدّ الذرة الشامية (*Zea mays L.*) من أهم محاصيل الحبوب في الدول النامية. ويُعدّ فطر *Magnaporthiopsis maydis* المسبِّب لمرض الذبول المتأخر في الذرة من أخطر الأمراض الفطرية التي تصيب هذا المحصول في مصر. وقد أظهرت عزلة المنيا-2 أعلى درجات الضراوة على صنف الذرة بوشي (*Boushy cv.*) تحت ظروف العدوى الحقلية.

و قد أوضحت النتائج أن نمو *M. maydis* على بيئة البطاطس ديكستروز آجار (PDA) انخفض بشدة عند معاملته بالكتوزان (Chitosan) وجسيمات الكيتوزان النانوية (Chitosan nanoparticles, NPs) بتركيزات 50، 100، 150، و200 جزء في المليون. في المقابل، لم يتأثر نمو الفطر *Trichoderma harzianum* بشكل واضح عند التركيزات 50 إلى 150 جزء في المليون، بينما انخفض نموه بشكل ملحوظ عند 200 جزء في المليون.

أظهرت صور المجهر الإلكترونى النافذ (TEM) أن أغشية خلايا الميسيليوم لكل من *M. maydis* و *T. harzianum* قد تعرضت لتغيرات تركيبية نتيجة المعاملة بالكتوزان وجسيماته النانوية. وأظهرت قطاعات الميسيليوم للفطر *M. maydis* حدوث تحلل جزئي في جدار الخلية وانكماش في الغشاء البلازمي. كما سبّبت جسيمات الكيتوزان النانوية تلفاً واضحاً في جدران وأغشية الخلايا مما أدى إلى موتها، في حين ظهرت خلايا الكونترول غير المعامل ذات عضيات محددة بوضوح.

وفي التجارب الحقلية وتحت ظروف االصوبة، أدّت معاملات *T. harzianum* منفرداً أو بالاشتراك مع الكيتوزان وجسيماته النانوية بتركيزات 50، 100، و150 جزء في المليون إلى تحسين مقاومة نباتات الذرة لمرض الذبول المتأخر وخفض نسبة الإصابة مقارنة بالكونترول. كما أدت هذه المعاملات إلى زيادة نشاط انزيمات الاكسدة في أوراق الذرة، مثل إنزيمات-β-1,3 جلوكاناز، والبيروكسيداز، والكاتالاز، وذلك مقارنة بكل من النباتات المصابة وغير المصابة.

وخلال موسم النمو لعام 2024، كانت معاملة *T. harzianum* مع جسيمات الكيتوزان النانوية بتركيز 150 جزء في المليون هي الأكثر فاعلية في زيادة نشاط الانزيمات، والحد من الإصابة بمرض الذبول المتأخر، وتحسين صفات النمو ومكونات المحصول في كلٍّ من محطتي الجمّيزة وملوي.